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DETECTION OF MINIMAL RESIDUAL DISEASE AND WT1 OVEREXPRESSION IN CHILDHOOD ACUTE LEUKAEMIA

A thesis submitted for the degree of
Doctor of Philosophy by

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August 2008

Declaration

I hereby certify that this thesis, submitted for the degree of Doctor of Philosophy to the University of Dublin, has not been previously submitted for a degree or diploma to this or any other university. The work presented here is entirely my own, except where stated.

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Acknowledgements

I would like to thank to all the people around me who have helped me so much and without whom not only I would never finish this PhD but probably never even started it. I have been grateful for their guidance, advice and assistance throughout my study, as well as for their so friendly support and encouragement at the times when nothing seemed to work.

Prof. Mark Lawler, my supervisor

Dr. Aengus O'Marcaigh, Prof. Owen Smith and Dr. Sean Rooney from Department of Haematology, Our Lady's Hospital for Sick Children, Crumlin, Dublin

Prof. Shaun McCann, Dr. Paul Browne, Dr. David O'Brien and Dr. Paul O'Rourke from Department of Haematology, St. James's Hospital and Trinity College Dublin

Prof. Dermot Kelleher from Institute of Molecular Medicine and Trinity College Dublin, and all the people from his working group

Dr. Fiona Quinn and Dr. Mireille Crampe from Cancer Molecular Diagnostic Department, St. James's Hospital, Dublin

Prof. Jan Trka and Prof. Ondrej Hrusak from Clinic of Paediatric Haematology and Oncology, University Hospital Motol and Charles University, Prague, and all the people from their working groups

Prof. Zdenek Sedlacek from Institute of Medical Biology and Genetics, Charles University, Prague

Prof. Jitka Abrahamova from Department of Oncology, Thomayer Teaching Hospital, Prague

Dr. Tony Fitzgerald from Haughton Institute, St. James's Hospital, Dublin, and Marek Omelka from Department of probability and mathematical statistics, Charles University, Prague

All my colleagues and friends from Department of Haematology and Oncology, Institute of Molecular Medicine, Trinity College Dublin

My family and friends

Finally, I would like to thank to the patients and their parents from Department of Haematology, Our Lady's Hospital for Sick Children, Crumlin, Dublin, for donation of the clinical samples.

This project was supported by the Higher Education Authority Programme for Research in Third Level Institutions (HEA PRTLI), Children's Leukemia Research Project, Children's Medical and Research Foundation Our Lady's Hospital for Sick Children in Crumlin (OLHSC), IGA (Czech Ministry of Health) 7439 and MSM (Czech Ministry of Education) 0021620813.

Publications:

Boublikova L., Kalinova M., Ryan J., Quinn F., O'Marcaigh A., Smith O., Browne P., Stary J., McCann S.M., Trka J., Lawler M.: Wilms' tumor gene 1 (WT1) expression in childhood acute lymphoblastic leukemia: a wide range of WT1 expression levels, its impact on prognosis and minimal residual disease monitoring. Leukemia (2006) 20, 254 – 263

Ryan J., Quinn F., Meunier A., Boublikova L., Crampe M., Tewari P., O'Marcaigh A., Stallings R., Neat M., O'Meara A., Breathnach F., Browne P., McCann S.M., Smith O., Lawler M.: A single centre study of molecular and immunophenotypic detection of minimal residual disease in childhood acute lymphoblastic leukaemia
- under submission

Presentations:

Boublikova L., Ryan J., Meunier A., O'Marcaigh A., Smith O., McCann S.M., Browne P., Lawler M.: Wilms' tumor gene 1 overexpression in childhood ALL (oral presentation). The Haematology Association of Ireland Annual Meeting, November 2004, Galway

Boublikova L., Kalinova M., Quinn F., Ryan J., Muzikova K., O'Marcaigh A., Browne P., Smith O., Stary J., McCann S.M., Trka J., Lawler M.: [Wilms' tumor gene 1 (WT1) expression in childhood ALL] (oral presentation, in Czech). Olomoucke Hematologicke Dny Conference, May 2006, Olomouc, Czech Republic

Boublikova L., Kalinova M., Ryan J., Quinn F., O'Marcaigh A., Smith O., Browne P., Stary J., McCann S.M., Trka J., Lawler M.: Wilms' tumor gene 1 (WT1) expression in childhood acute lymphoblastic leukemia (poster). The European Hematology Association 11th Congress, June 2006, Amsterdam, The Netherlands; abstract included in: Haematologica (2006), 91 (s1), 242

Abstract

Minimal residual disease (MRD), the post-treatment presence of a small submicroscopic amount of leukaemic cells in the bone marrow or other sites undetectable by conventional methods, has been recently shown to be one of the most important independent prognostic factors in childhood acute leukaemia. MRD can be detected by immunophenotyping by flow cytometry, PCR of chromosomal aberrations and PCR of clonal immunoglobulin or T-cell receptor (*IG/TCR*) rearrangements; each of these methods having certain advantages, disadvantages and limitations. In this study, which is a part of a larger MRD project, three different methods of MRD detection were first established, optimised, and validated according to the BIOMED-1 and Europe Against Cancer Program recommendations, and then used to monitor the MRD status of a group of 25 patients with childhood acute leukaemia (23 ALL, 2 AML) during at least 1-year follow-up. In 4 of the 23 ALL patients (17 %) MRD could be followed by only one method – half of them by FC, the other half by PCR of clonal *IG/TCR* rearrangements. Combined monitoring by FC and PCR (of chromosomal aberration or clonal *IG/TCR* rearrangements) was applicable in 7 patients (30 %) and all three approaches of MRD detection could be used in 5 patients (22 %). In the remaining 7 ALL patients enrolled in the study (30 %), MRD could not be monitored because either diagnostic of follow-up samples were missing. The 2 AML patients did not express any of the screened chromosomal aberrations and both were monitored by FC only. The correlation of MRD presence and levels detected by different targets within and in between the several methods showed a very good overall correlation with $p < 0.0001$ in Spearman rank test. Though certain differences could be noticed, the individual discordances were rare and confined to low levels of MRD (mostly below 10^{-3} or 0.1 %). Of the 16 monitored ALL patients, 7 (44 %) reached MRD negativity by day 29, and all of them had MRD levels on day 15 below 0.1 % of the initial values in their diagnostic samples, which classified them into MRD low-risk group. On the other hand, 3 patients (19 %) achieved MRD negativity only after week 12; 2 of them had also very high MRD levels in day 15 and day 29 samples (over 10 % and 0.1 %, respectively). These patients belonged to MRD high-risk group. Of the remaining patients, 5 (31 %) became MRD negative between day 29 and week 12, thus belonging to MRD intermediate risk group. One patient (6 %) had no day 29 sample available, all his later samples were MRD negative. As no case of relapse appeared, probably due to the limited number of patients and length of follow-up, no conclusion on the prognostic value of the measured MRD levels and MRD risk stratification in our patients could be drawn at this moment.

In the second part of the project, a search for novel potential prognostic factors and MRD markers was performed and focused on Wilms' tumour gene 1 (*WT1*) expression in acute leukaemia. *WT1*, located on chromosome 11p13, encodes a zinc-finger transcription factor with important roles in embryogenesis and oncogenesis. It is supposed to be overexpressed in the majority (70 – 90 %) of acute leukaemias and has been identified as an independent adverse prognostic factor, a convenient MRD marker and potential therapeutic target in acute leukaemia. Nevertheless, its clinical impact in childhood acute leukaemia, particularly ALL, remains unclear. Using RT-PCR and RQ-PCR for detection of total *WT1* and its main isoforms of our own design, which were optimised in a similar way as the methods for MRD detection,

we evaluated *WT1* in diagnostic bone marrow (BM) samples of 128 consecutively enrolled childhood acute leukaemia patients (107 B-ALL, 19 T-ALL, 2 AML) and compared it with physiologic *WT1* expression in normal and regenerating BM and normal peripheral blood (PB). In B-ALL, we detected a wide range of *WT1* levels (5 logs) with median *WT1* expression close to that of normal bone marrow. *WT1* expression in T-ALL was significantly higher than in B-ALL ($p < 0.001$). In 3 paired BM and PB samples of B-ALL patients, *WT1* levels in PB were approximately 1 log lower than in the corresponding BM. In AML, normal or increased *WT1* expression was detected. All four *WT1* isoforms were found in normal controls and leukaemia samples. In a subanalysis of ALL patients, those with *MLL-AF4* translocation showed high *WT1* overexpression ($p < 0.01$) compared to patients with other or no chromosomal aberrations. Older children (≥ 10 years) expressed higher *WT1* levels than children under 10 years of age ($p < 0.001$), while there was no difference in *WT1* expression in patients with peripheral blood leukocyte count (WBC) $\geq 50 \times 10^9$ / l and lower. Analysis of relapsed cases (14/125) indicated that abnormal increase or decrease in *WT1* expression was associated with significantly increased risk of relapse ($p = 0.0006$), and this prognostic impact of *WT1* was independent of other main risk factors ($p = 0.0012$). In summary, this part of the study suggests that *WT1* expression in childhood ALL is very variable and much lower than reported in AML or adult ALL. *WT1* thus will not be a useful marker for MRD detection in childhood ALL, however, it does represent a potential independent risk factor in childhood ALL. Interestingly, a proportion of childhood ALL patients express *WT1* at levels below the normal physiological bone marrow *WT1* expression, and this reduced *WT1* expression appears to be associated with a higher risk of relapse, which has not been described previously.

Thus using these approaches, the relevance of flow cytomeric and molecular analysis of minimal residual disease in childhood acute leukaemia has been verified and the potential for a new prognostic marker in this disease has been indicated.

Keywords: childhood acute leukaemia; minimal residual disease (MRD); flow cytometry; RT-PCR; real-time quantitative PCR (RQ-PCR); chromosomal aberrations; immunoglobulin and T-cell receptor clonal rearrangements; Wilms' tumour gene 1 (*WT1*); prognostic factor

Glossary

ABL	Abelson gene
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ANLL	acute non-lymphoblastic leukaemia
APL	acute promyelocytic leukaemia
ATRA	all-trans-retinoic acid
B2M	β-2-microglobulin
BM	bone marrow
chAL	childhood acute leukaemia
CG	control gene
CML	chronic myeloid leukaemia
CR	complete remission
FC	flow cytometry
FG	fusion gene
IFM	immunofluorescence microscopy
IG	immunoglobulin
IHC	immunohistochemistry
LAP	leukaemia-associated phenotype
MDS	myelodysplastic syndrome
MRD	minimal residual disease
NCN	normalised copy number (FG/CG or WT1/CG)
OS	overall survival
PB	peripheral blood
PCR	polymerase chain reaction
PSC	peripheral stem cells
RA	retinoic acid
RQ-PCR	real-time quantitative PCR
RT	reverse transcription
t-AML	therapy-related AML
TCR	T-cell receptor
WBC	white blood cell count
WT1	Wilms' tumour gene 1

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1. Introduction

Acute leukaemia is the most frequent paediatric malignancy with increasing incidence in developed countries. Its prognosis has improved dramatically in the last two decades, with 70 - 80 % of children with acute lymphoblastic leukaemia (ALL) and over 50 % of children with acute myeloid leukaemia (AML) achieving long term complete remission, an outstanding result of treatment among all malignant diseases. However, 20 - 30 % of patients with childhood ALL and up to 50 % of patients with childhood AML still die of this disease at present. In addition, a substantial proportion of acute childhood leukaemia survivors may suffer from late side effects of the antileukaemic therapy negatively influencing their quality of life or even shortening their life expectancy.

The present management of childhood acute leukaemia (chAL) therefore follows two main goals: to further increase the cure rates and, at the same time, to minimise the late consequences of the therapy. The effective antileukaemic therapy aims to induce haematological remission and prevent later relapses while avoiding treatment-related complications. In practice, these goals may be achieved by highly individualised risk-directed therapy, and by development of new modes of efficient and specific antileukaemic therapy.

Childhood acute leukaemia, particularly ALL, represents a biologically heterogenous group of diseases with varying responses to the treatment and prognosis. As the intensity and toxicity of the conventional modalities of antileukaemic treatment (chemotherapy, radiotherapy, stem cell transplantation) are tightly related, the intensity and consequently the toxicity of the therapy should be adjusted to and reflect the individual patient's and disease risks. The prediction of the probable disease course, treatment response and patient's prognosis is based on assessment of prognostic and predictive factors. These factors include a group of patients' characteristics at diagnosis (e.g. age, sex, race), disease's characteristics at diagnosis (e.g. leukocyte count, organ involvement, cytogenetic and molecular abnormalities) and early response to the initial therapy (that may be evaluated from peripheral blood morphology, bone marrow morphology, and recently by minimal residual disease detection). According to such risk factors, patients are stratified into groups with similar prognosis and treated with adequate protocols.

At present, even the most intensive treatment consisting of combination of chemotherapy and radiotherapy with or without stem cell transplantation and with massive supporting care cannot cure a proportion of patients with resistant disease. New treatment approaches, like immunotherapy and molecular therapy, promise an efficient and highly selective antileukaemic effect and should further improve the treatment outcomes.

The current progress and success in chAL management are closely associated with the extensive developments and advances in leukaemia research. Our understanding to the molecular basis of haematological malignancies, signalling pathways, regulation of cell proliferation, interactions and death, the process of leukaemogenesis and the effect of different factors on cellular functions have contributed enormously to chAL diagnosis and therapy, and provide new impetus for its prevention. New techniques in molecular biology, cytogenetics and immunology enable recognition of specific disease entities and

subgroups that have a particular biologic behaviour correlating with certain prognosis and response to the therapy. They also enable identification of immunological and molecular abnormalities in malignant cells that may act as potential therapeutic targets.

One of the significant new findings of leukaemia research with considerable clinical implications has been the definition and diagnosis of minimal residual disease (MRD). MRD refers to the posttreatment presence of a small submicroscopic amount of leukaemic cells in the bone marrow or other sites undetectable by conventional methods. Three main techniques can be used for MRD detection: immunophenotyping by flow cytometry, PCR of chromosomal aberrations and PCR of clonal immunoglobulin or T-cell receptor (*IG/TCR*) rearrangements, each of these methods having certain advantages, disadvantages and limitations. In a number of large prospective studies MRD has been shown to be one of the most important independent prognostic factors in chAL, but only recently it has been integrated into international treatment protocols. Despite its potential key role in chAL management, there are many questions and issues around MRD that have yet to be answered, including the type of sample investigated (bone marrow, peripheral blood), the amount of cells analysed, the most significant time points for MRD detection, the methods used for MRD monitoring and their parameters.

Presently, a considerable effort is focused on increasing efficiency, simplification and standardisation of the tests for MRD detection, so as they can be used not only in specialised research centres but in the setting of a routine clinical laboratory, making them available for all patients diagnosed with chAL. The standardisation of the techniques should also improve the reproducibility of the results, their comparison between different centres, and is an essential prerequisite for large-scale multiinstitutional and international studies, which only have the potential to reveal the real role and significance of MRD in various leukaemia types and subtypes. At the same time, a search for new potential MRD markers and development of new methods of MRD detection continue and several of these, like PCR detection of Wilms' tumour gene 1 overexpression, detection of clonal *IG/TCR* rearrangements by fluorescent PCR with genescan analysis, and global gene expression profiling by gene microarrays, have already shown a lot of promise. They could offer higher sensitivity and specificity, broader applicability, reduction of labour and costs in MRD detection, and may also identify new molecules and pathways involved in the leukaemogenic process that may be, apart from being an MRD marker, also a suitable target for a specific molecular therapy.

2. Background

2.1. Leukaemogenesis and molecular biology of childhood acute leukaemia

2.1.1. Malignant transformation of the haematopoietic cell

Acute leukaemia arises as a result of severe and irreversible dysregulation of haematopoietic developmental programmes. The disruption of normal regulatory mechanisms is caused by certain genetic alterations, mutations, and may also involve certain epigenetic alterations. The biological features of malignant leukaemic cells, like immortality, permanent proliferation with inhibition of differentiation and apoptosis, invasiveness, independence on stimulatory factors and lack of response to control mechanisms, are phenotypic manifestations of these alterations [Knowles *et al* 2001, Hoffbrand *et al* 1999, Alcalay *et al* 2001].

All leukaemic cells arise from a pluripotent haematopoietic precursor that has undergone a malignant transformation. The malignant transformation of a normal stem cell into a malignant leukaemic stem cell is a multistep process during which successive oncogenic mutations affecting at least several critical genes occur. These mutations are either gain-of function or loss-of-function mutations and affect two principal groups of genes: protooncogenes and tumour-suppressor genes. An accumulation of the mutations leads to progressive dysregulation of the principal cellular functions, proliferation, differentiation and apoptosis, leading to the development of a malignancy. The number of genetic alterations necessary for the development of acute leukaemia is still a matter of debate. Though products of certain single gene rearrangements have been shown to induce acute leukaemia in animal models, it seems that several genetic alterations affecting both, proliferation (dysregulation leading to hyperproliferation and lack of response to inhibitory signals) and differentiation and apoptosis (inhibition) are probably necessary to induce acute leukaemia [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, McKinnell *et al* 1998].

During the process of malignant transformation, a normal haematopoietic precursor changes into a precursor displaying various abnormalities in proliferation, differentiation or survival (hyperproliferation, dysplastic features, suppression of apoptosis) but still responding to regulatory mechanisms, and this cell consequently transforms into a leukaemic precursor with full malignant features, including uncontrolled, independent and invasive growth pattern.

The haematopoietic cell must be permissive for expression of the mutation. The transforming functions of the mutations are often cell-context dependent, which explains why mutations at the level of pluripotent stem cells manifest in distinct lineage-affiliated cell phenotypes and give rise to distinct subtypes of acute leukaemia [Greaves *et al* 2003].

There is compelling evidence that some of the mutations associated with paediatric leukaemia originate prenatally *in utero* during foetal haematopoiesis [Biondi *et al* 2000a, Greaves *et al* 2003]. In most cases, further postnatal mutational events are necessary for the development of acute leukaemia.

Leukaemia has a clonal character, all leukaemic cells are derived from a single original leukaemic precursor (a clonal precursor) with the initiating mutations. The clonal precursor is a haematopoietic cell at the earliest stages of differentiation, the pluripotent stem cell or the common myeloid or common lymphoid precursor. The transforming mutations are usually non-random, typically and predominantly associated with special subtypes of leukaemia. Proliferation of the leukaemic precursor gives rise to a clone of leukaemic cells. The establishment of this leukaemic clone means the onset of the disease, leukaemia, and its further proliferation leads to the clinical manifestation of leukaemia.

The process of genetic alterations by successive mutations may continue during the progress of the malignant disease. These further mutational events occur in cells of the proliferating leukaemic clone and, although the genomic stability of haematological malignancies is in general much higher than in solid tumours, they consequently lead to a certain heterogeneity of the population and clonal diversification. Leukaemic cells that have acquired new mutations bringing them a growth advantage may overgrow the others and suppress their proliferation. The formation and selection of subclones of leukaemic cells with partially different, more aggressive features that gradually predominate and replace the original cells is called clonal progression or clonal evolution. The clonal progression may be responsible for changes in cytogenetic and molecular markers of the leukaemic blasts, the disease acceleration and decreasing sensitivity to antileukaemic therapy. The subclone selection occurs as a result of natural selective pressures (space, nutrients, negative regulators) during the disease progression, but is also considerably enhanced by antileukaemic treatment, eradicating sensitive and thus perhaps selecting resistant leukaemic cells [Knowles *et al* 2001, Hoffbrand *et al* 1999, Hoffbrand *et al* 2001] (Figure 2.1.).

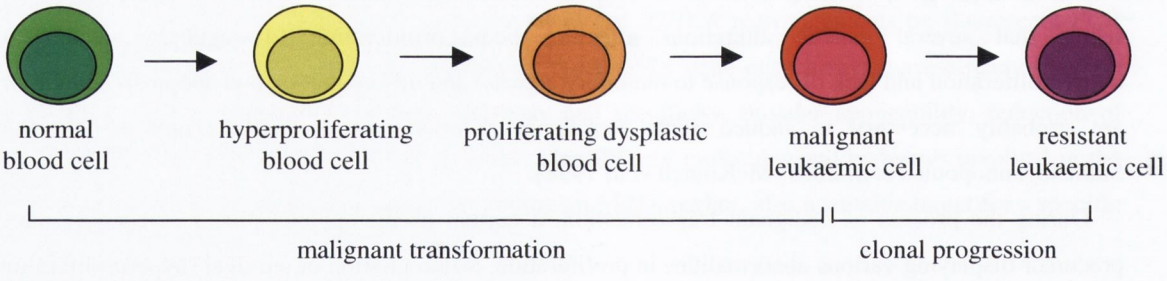


Figure 2.1. The process of malignant transformation and clonal progression.

The kinetics of proliferation of the leukaemic cell population differs considerably from normal population of haematopoietic cells, and undergoes changes during the course of the disease. In leukaemic population, the proliferative fraction (the rate between proliferating cells and non-proliferating cells) is high. This is the reason for the rapid expansion of the leukaemic population and its ability to overgrow normal haematopoietic cells, despite the fact that leukaemic cells usually have longer cell cycle than normal cells. At the beginning of the disease, shortly after the malignant transformation, practically all

cells of the leukaemic clone proliferate and their number increases exponentially. As the total population of leukaemic cells expands, the fraction of proliferating cells decreases, doubling time (time during which the cell population doubles in its number) prolongs and more non-proliferating leukaemic cells appear. This may be a result of acquiring mutations that put a block on further proliferation or may reflect the inconvenient surrounding environment (lack of nutrient substrates, limited space, effect of antitumour therapy). The non-proliferating leukaemic cells are represented by cells that are in a quiescent phase but retain their ability to return to the cell cycle and proliferate, cells that have lost the proliferative potential and dying cells. Most of the disease development occurs asymptomatically, in the pre-clinical period. When leukaemia clinically manifests, the total number of leukaemic cells in the body reaches 10^{11} - 10^{12}. Even after complete remission has been achieved the number of residual leukaemic cells stays high, around 10^{10} - 10^{11} [Degos *et al* 2000, Hoffbrand *et al* 1999, Hoffbrand *et al* 2001, McKinnell *et al* 1998].

The malignant disease, leukaemia, is characterised by the presence of a clone of leukaemic cells. During life of an individual, single cells with malignant leukaemic characteristics may occur in the body. They either do not have enough potential to give rise to a clone of daughter malignant cells and consequently die, or are recognised by the effector cells of the immune system and eradicated. In both cases they do not lead to the development of leukaemia.

In very rare cases, some malignant diseases may show spontaneous remission. The malignant cells either differentiate and lose the malignant properties, or the malignant clone disappears due to acquiring genetic alterations inconsistent with further survival. This is a unique feature of some childhood malignant diseases, in haematology for example transient myeloproliferative syndrome in neonates with Down's syndrome [Knowles *et al* 2001, Degos *et al* 2000, Hoffbrand *et al* 1999, www.nci.nih.gov].

2.1.2. Typical cytogenetic and molecular alterations in childhood acute leukaemia

Many molecular changes have been well characterised in acute leukaemia and further are being identified. However, only few of this large group of cytogenetic and molecular alterations occur with frequency > 5 %, the vast majority of them are quite rare, occurring in < 1 % of acute leukaemia cases. They involve numerical or structural changes that occur in leukaemia in recurrent patterns. Some of these genetic lesions are more general and can be found in both ALL and AML and their different subtypes, as well as in other haematological malignancies. Others are very specific for or predominantly associated with a defined acute leukaemia type and subtype. In childhood acute leukaemia, balanced translocations predominate. The only exception is secondary leukaemia with prevailing numerical and unbalanced abnormalities. In some cases, a coincidence of several genetic alterations may be detected, but complex aberrations (more than 3 abnormalities) are rather rare, usually associated with an advanced disease and poor prognosis [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, Hall *et al* 2001, Grimwade *et al* 2001, Biondi *et al* 2000a, Dash *et al* 2001, Anastasiadou *et al* 2003, Qiao *et al* 2003, Moppett *et al* 2003, Greaves *et al* 2003] (Table 2.1.).

All the molecular alterations can be detected by PCR-based molecular biologic methods. Many of them may be also detected with less sensitive techniques, like standard cytogenetic tests and FISH (fluorescence in-situ hybridization), or immunological methods (flow cytometry, immunocytology).

Study of these molecular abnormalities is important for several reasons. They often show a strong correlation with etiopathogenic, immunophenotypic and clinical features and possess considerable prognostic implications. They may enable identification of leukaemic cells present in very low amounts and be convenient markers of minimal residual disease. And finally, many of them are directly involved in the process of acute leukaemia development and thus represent potent targets for both therapy and prevention of acute leukaemia.

Table 2.1. Molecular abnormalities in childhood acute leukaemia – cont.

Molecular alterations in childhood acute leukaemia			
1. Alterations with dysregulated gene expression			
Alteration	Genes involved	Character of the genes	Disease
t(5;14)(q31;q32)	IL-3 – IgH	GF – Ig	B-ALL
t(1;7)(p34;q34)	LCK – TCRβ	RTK – TCR	T-ALL
t(8;14)(q24;q32)	MYC – IgH	TF(bHLH-LZ) – Ig	B-ALL
t(2;8)(p12;q24)	Igκ – MYC	Ig – TF(bHLH-LZ)	B-ALL
t(8;22)(q24;q11)	MYC – Igλ	TF(bHLH-LZ) – Ig	B-ALL
t(8;14)(q24;q11)	MYC – TCRα/δ	TF(bHLH-LZ) – TCR	T-ALL
t(1;7)(p32;q35)	TAL1 – TCRβ	TF(bHLH) – TCR	T-ALL
t(1;14)(p32;q11)	TAL1– TCRα/δ	TF(bHLH) – TCR	T-ALL
del(1)(p32)	SIL – TAL1	– TF(bHLH)	T-ALL
t(1;3)(p34;q21)	TAL1 – TCTA	TF(bHLH) –	T-ALL
t(7;9)(q35;q34)	TCRβ – TAL2	TCR – TF(bHLH)	T-ALL
t(7;19)(q35;p13)	TCRβ – LYL1	TCR – TF(bHLH)	T-ALL
t(11;14)(p15;q11)	TTG1– TCRα/δ	TF(LIM) – TCR	T-ALL
t(11;14)(p13;q11)	TTG2 – TCRα/δ	TF(LIM) – TCR	T-ALL
t(7;11)(q35;p13)	TCRβ – TTG2	TCR – TF(LIM)	T-ALL
t(7;10)(q35;q24)	TCRβ – HOX11	TCR – TF(HD)	T-ALL
t(10;14)(q24;q11)	HOX11 – TCRα/δ	TF(HD) – TCR	T-ALL
inv(14)(q11;q32) t(14;14)(q11;q32)	TCRα/δ – TCL1	TCR –	T-ALL
t(5;14)(q35;q32)	HOX11L2 – CTIP2	TF(HD) –	T-ALL
t(x;14)(q28;q11)	MTCP1(C6.1B) – TCRα/δ	– TCR	T-ALL
inv(3)(q21;q26) t(3;3)(q21;q26)	x – EVI1	ribophorin – TF(ZF)	AML
t(1;3)(p36;q21)	x – MEL1	– TF(ZF)	AML

Table 2.1. Molecular abnormalities in childhood acute leukaemia – cont.

2. Alterations with fusion gene formation or gene structure alteration			
Alteration	Genes involved	Character of the genes	Disease
itd(13q12) 13q12 mutations	Flt3	RTK	AML, ALL
4q12 mutations	c-kit	RTK	AML
microdel1q21	TRKA	RTK	AML
t(5;14)(q33;q32)	PDGFβR – CEV14	RTK –	AML
t(8;13)(p12;q12)	FGFR1– ZNF198	RTK – TF(ZF)	AML
t(8;9)(p12;q33)	FGFR1– CEP110	RTK – sp	AML
t(6;8)(q27;p12)	FOP – FGFR1	rp – RTK	AML
t(9;12)(p24;p13)	JAK2 – TEL	RTK – TF(ETS)	ALL
t(9;22)(q34;q11)	ABL – BCR	NRTK – S/TK	ALL, AML
t(9;12)(q34;p13)	ABL – TEL	NRTK – TF(ETS)	AML, ALL
t(7;9)(q34;q34)	TCRβ – NOTCH1(TAN1)	TCR – R	T-ALL
t(1;19)(q23;p13)	PBX1 – E2A	TF(HD) – TF(bHLH)	B-ALL
t(17;19)(q22;p13)	HLF – E2A	TF(LZ) – TF(bHLH)	B-ALL
t(7;12)(q32;p13)	HOXB9 – TEL	TF(HD) – TF(ETS)	ALL, AML
t(7;12)(q36;p13)	HLXB9 – TEL	TF(HD) – TF(ETS)	AML
t(1;12)(q21;p13)	ARNT – TEL	– TF(ETS)	AML
t(1;12)(q25;p13)	ARG – TEL	NRTK – TF(ETS)	AML
t(4;12)(q11-12;p13)	BTL – TEL	– TF(ETS)	AML
t(5;12)(q31;p13)	ACS2 – TEL	– TF(ETS)	AML
t(9;12)(q22;p13)	SYK – TEL	NRTK – TF(ETS)	AML
t(12;13)(p13;q12)	TEL – CDX2	TF(ETS) – TF(HD)	AML
t(12;13)(p13;q14)	TEL –TTL	TF(ETS) –	ALL
t(12;15)(p13;q25)	TEL – TRKC	TF(ETS) – RTK	AML
t(9;12)(q11;p13)	PAX5 – TEL	TF(HD-ZF) – TF(ETS)	ALL
t(3;12)(q26;p13)	MDS1/EVI1 – TEL	TF(ZF) – TF(ETS)	AML
t(12;22)(p13;q11)	TEL – MN1	TF(ETS) –	AML
t(12;21)(p13;q22)	TEL – AML1	TF(ETS) – TF(RUNT)	B-ALL
t(8;21)(q22;q22)	ETO – AML1	CF – TF(RUNT)	AML
t(3;21)(q26;q22)	EAP – AML1	– TF(RUNT)	AML
t(3;21)(q26;q22)	MDS – AML1	– TF(RUNT)	AML
t(3;21)(q26;q22)	EVI1 – AML1	TF(ZF) – TF(RUNT)	AML
t(16;21)(q24;q22)	MTG16 – AML1	– TF(RUNT)	AML
inv(16)(p13;q22) t(16;16)(p13;q22) del(16)(q22)	MYH11 – CBFB	sp – CF	AML
t(15;17)(q22;q21)	PML – RARA	TF(ZF) – LTF(ZF)	AML
t(11;17)(q23;q21)	PLZF – RARA	TF – LTF(ZF)	AML
t(5;17)(q35;q21)	NPM – RARA	nucleophosmin -LTF(ZF)	AML
t(11;17)(q13;q21)	NUMA1 – RARA	sp – LTF(ZF)	AML
der(17)	STAT5B – RARA	RTK – LTF(ZF)	AML
t(4;11)(q21;q23)	AF4 – MLL	TF – TF(AT-ZF)	ALL, AML
t(9;11)(p21;q23)	AF9 – MLL	TF – TF(AT-ZF)	ALL, AML
t(11;19)(q23;p13.3)	MLL – ENL	TF(AT-ZF) – TF	ALL, AML
t(11;19)(q23;p13.1)	MLL – ELL	TF(AT-ZF) – CF	AML
t(11;19)(q23;p13)	MLL – ENN	TF(AT-ZF) – rp	AML
t(1;11)(q21;q23)	AF1q – MLL	rp – TF(AT-ZF)	AML
t(1;11)(p32;q23)	AF1p –MLL	TF – TF(AT-ZF)	ALL, AML
t(5;11)(q31;q23)	GRAF – MLL	– TF(AT-ZF)	AML
t(5;11)(q31.1;q23) ins(5;11)(q31;q13q23)	AF5q31 – MLL	– TF(AT-ZF)	AML
t(6;11)(q25;q23)	AF6 – MLL	rp – TF(AT-ZF)	ALL, AML

Table 2.1. Molecular abnormalities in childhood acute leukaemia.

2. Alterations with fusion gene formation or gene structure alteration – cont.			
Alteration	Genes involved	Character of the genes	Disease
t(6;11)(q21;q23)	AF6q21 – MLL	TF – TF(AT-ZF)	AML
t(11;17)(q23;q21)	MLL – AF17	rp – TF(LZ-ZF)	AML
ins(11;9)(q23;q34)	MLL – FBP17	– TF(LZ-ZF)	AML
t(10;11)(p11;q23)	MLL – ABI1	TF(AT-ZF) – rp	AML
t(11;22)(q23;q11.2)	MLL – hCDCrel1	TF(AT-ZF) – rp	AML
t(11;22)(q23;q13)	MLL – P300	TF(AT-ZF) – CF	AML
t(11;16)(q23;p13)	MLL – CBP	TF(AT-ZF) – CF	AML
t(11;17)(q23;q25)	MLL – MSF/AF17q25	TF(AT-ZF) – rp	AML
t(11;14)(q23;q24)	MLL – h.gephyrin	TF(AT-ZF) –	AML
t(11;15)(q23;q14)	MLL – AF15q14	TF(AT-ZF) –	AML
t(11;17)(q23;p13)	MLL – GAS7	TF(AT-ZF) –	AML
ins(X;11)(q24;q23)	Septin 6 – MLL	TF(AT-ZF) –	AML
microdel(11q23)	MLL	TF(AT-ZF)	T-ALL
itd(11q23)	MLL	TF(AT-ZF)	AML, ALL
t(6;9)(p23;q34)	DEK – CAN	CF – nucleoporin	AML
t(9;9)(q43;q34)	SET – CAN	– nucleoporin	AML
t(7;11)(p15;p15)	HOXA9 – NUP98	TF(HD) – nucleoporin	AML
t(2;11)(q35;p15)	HOXD13 – NUP98	TF(HD) – nucleoporin	AML
t(11;12)(p15.5;q13)	NUP98 – HOXC13	nucleoporin – TF(HD)	AML
inv(11)(p15;q22)	NUP98 – DDX10	nucleoporin -RNA helicase	AML
t(1;11)(q23;p15)	PMX1 – NUP98	TF(HD) – nucleoporin	AML
t(5;11)(q35;p15.5)	NSD1 – NUP98	– nucleoporin	AML
t(9;11)(p22;p15.5)	LEDGF – NUP98	GF – nucleoporin	AML
t(11;20)(p15.5;q11)	NUP98 – TOP1	nucleoporin –	AML
t(4;11)(q21;p15.5)	RAP1/GDS1 – NUP98	– nucleoporin	T-ALL
t(1;22)(p13;q13)	OTT – MAL	– TF	AML
t(8;16)(p11;p13)	MOZ – CBP	TF(ZF) – CF	AML
t(8;22)(p11;q13)	MOZ – P300	TF(ZF) – CF	AML
inv(8)(p11;q13)	MOZ – TIF2	TF(ZF) –	AML
t(10;16)(q22;p13)	MORF – CBP	– CF	AML
t(16;21)(p11;q22)	FUS – ERG	– TF(ETS)	AML
t(3;5)(q35;q35)	NPM – MLF1	nucleophosmin –	AML
t(10;11)(p13;q14)	CALM – AF10	rp –	AML, ALL
12p mutations	K-Ras, N-Ras	GBP	AML, ALL
3. Alterations with loss of gene expression			
Alteration	Genes involved	Character of the genes	Disease
9p21	p16	TSG	ALL, AML
9p21	p15	TSG	ALL, AML
17p	p53	TSG	ALL, AML
13q14	RB	TSG	ALL, AML
11q22-23	ATM	TSG	T-ALL
7q	NF-1	rp(GAP)	AML

(GF – growth factor, RTK – receptor or receptor-associated tyrosine kinase, NRTK – non-receptor tyrosine kinase, R – receptor, S/TK – serine/threonine protein kinase, GBP – GTP-binding protein, TF – trancription factor, LTF – ligand-activated transcription factor, CF – co-factor of transcription factors or related to transcription, TSG – tumour suppressor gene, bHLH – basic helix-loop-helix, LZ –leucine zipper, ZF – zinc finger, HD homeodomain, ETS – Ets domain, RUNT – Runt homology domain, LIM – cystein-rich LIM domain, AT – A-T hook domain, rp- regulatory protein, sp – structural protein, itd – internal tandem duplication)

2.1.2.1. BCR-ABL translocation

BCR-ABL translocation is probably the best described chromosomal alteration. It is a pathognomic feature of chronic myeloid leukaemia but is also present in a proportion of precursor B-ALL cases. In childhood B-ALL it can be found only in about 5 % of patients, in adult B-ALL is much more frequent (20 – 50 %); its incidence in B-ALL increases with age. Rarely (in 1 - 2 % of cases) this translocation has been detected in AML [Knowles *et al* 2001, van Dongen *et al* 1999].

In this translocation, t(9;22)(q34;q11), the *BCR* gene on chromosome 22 is juxtaposed to the *ABL* gene on chromosome 9, forming a chimeric *BCR-ABL* gene. In more than 95 % of the cases, the translocation can be detected as the Philadelphia (Ph1) chromosome by standard cytogenetic techniques.

ABL, Abelson oncogene, encodes for a tyrosine kinase located predominantly in the nucleus but also present in the cytoplasm. It contains three SRC homology (SH) domains, SH1, SH2 and SH3, at its N-terminal portion, and DNA and actin binding domains at the C-terminal portion. The SH1 domain exhibits the tyrosine kinase activity and may be negatively regulated by SH3 domain. The first 26 or 45 amino acids of ABL are encoded by alternative exons 1a or 1b, respectively. BCR is a ubiquitously expressed cytoplasmic protein. It contains a SH2 binding domain, which overlaps with a serine/threonine protein kinase domain and includes coiled-coil motif at the N-terminal portion, a central domain with homology to guanine nucleotide exchange proteins, and a domain of GTPase activating protein (GAP, a member of the Ras-related small GTPase family) at the C-terminal portion.

The breakpoints are located in the *ABL* gene at amino acid 26 of type 1a or amino acid 45 of type 1b exons. The breakpoints in the *BCR* gene are grouped into two main regions: the major breakpoint cluster region (*M-BCR*), located in the central region of the BCR gene, or the minor breakpoint cluster region (*m-BCR*), located 40 kb upstream of the major breakpoint region. In a very small percentage of cases, the breakpoint region is localised downstream of the M-BCR, in so called micro breakpoint cluster region (*μ-BCR*). Sporadic cases with *ABL* or *BCR* breakpoints outside the defined regions have also been described. Depending on the breakpoint region within the *BCR* gene, different *BCR-ABL* junctions occur, producing fusion proteins of different molecular weight. The larger BCR-ABL p210 (210 kD) chimeric protein is produced as a result of breakpoints grouped in the *M-BCR*, fusing *BCR* exons b2 or b3 to *ABL* exon a2. Breakpoints that occur in the *m-BCR* lead to the production of the smaller BCR-ABL p190 (190 kD) chimeric protein with e1-a2 fusion. In childhood B-ALL, the p190 protein is found predominantly, in 70 - 80 % of cases. (In adult B-ALL p190 and p210 proteins are found in equal frequency, around 50 %; in CML the absolute majority of cases express the p210 protein.) (Figure 2.2.) Breakpoints within the *μ-BCR* lead to c3-a2 fusion gene encoding a large p230 fusion protein (230 kD). It shows similarities to p210 protein and is found in rare CML cases, where it may be associated with more indolent course of the disease (chronic neutrophilic leukaemia, CNL). Very rarely, *BRC* exons are fused to a3 exon of *ABL* [Knowles *et al* 2001, van Dongen *et al* 1999, Harrison *et al* 2000, 2001 and 2002].

p210 and p190 BCR-ABL proteins contain the same ABL-derived part but differ in the BCR-derived part. In both cases the almost entire ABL coding sequence with all its functional domains is fused to the N-terminal SH2-binding and serine/threonine kinase domains of BCR with (in case of p210) or without (in case of p190) the homology to guanine nucleotide exchange proteins region. The ABL kinase and the

BCR sequences are essential for the transforming abilities of BRC-ABL proteins. The two BCR-ABL fusion proteins, p190 and p210, show differences in biological activities but both have the oncogenic potential that has been demonstrated *in vitro* and *in vivo*. BCR-ABL fusion proteins exhibit a constitutive activation of the ABL tyrosine kinase activity. The oligomerization of the BCR-ABL protein into tetramers, mediated by the coiled-coil domain at the N-terminal of BCR, activates the tyrosine kinase activity of the normally silent c-ABL product and also leads to an extensive autophosphorylation of BCR. The interaction between SH2-binding domain of BCR and the joined SH2 domain of ABL inhibits the negative regulatory activity of the SH3 domain on the tyrosine kinase resulting in a permanent tyrosine kinase activity. Through protein-protein interactions mediated by multiple functional domains of both partner gene origins, BCR-ABL can bind to and phosphorylate different effectors and stimulate signal transduction pathways important for regulating many cellular functions. Unlike ABL, BCR-ABL has a cytoplasmic location, which may contribute to its aberrant function [Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, van Dongen *et al* 1999, Pane *et al* 2002, Anastasiadou *et al* 2003, Ravandi *et al* 2002, Scheijen *et al* 2002].

The leukaemogenic potential of BCR-ABL results from its ability to stimulate proliferation, inhibit differentiation and apoptosis and increase the genetic instability of the cells.

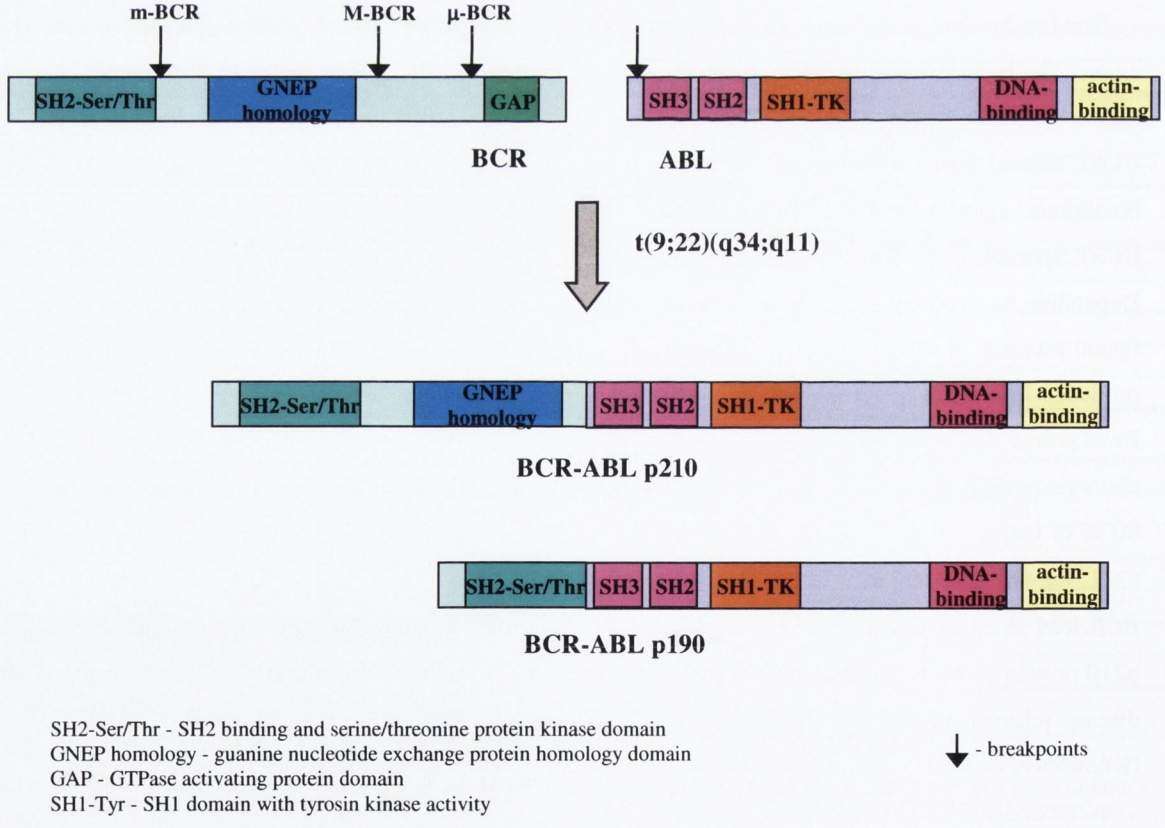


Figure 2.2. BCR-ABL translocation.

Upon autophosphorylation, BCR-ABL binds to the SH2 domain of the Grb-2 adapter protein. This interaction leads to recruitment and tyrosine phosphorylation of the scaffolding adapter Gab-2 (growth factor receptor-bound protein 2-associated binding protein), that interacts and activates SHP2 (SH2 domain containing protein tyrosine phosphatase), Ras-MAPK(Erk) and PI3K-Akt signalling pathways. BCR-ABL also acts through c-Myc signalling. The final result of activation of these pathways is the up-regulation of cyclin D2 and down-regulation of cyclin-dependent kinase inhibitor p27, causing deregulation of the cell cycle and increased proliferation [Ferrando *et al* 2000, Anastasiadou *et al* 2003, Scheijen *et al* 2002, Ravandi *et al* 2002].

BCR-ABL induced differentiational arrest is hypothesised to be due to the down-regulation of G-CSF receptor through down-regulation of C/EBPα, a principle transcription factor in granulocytic differentiation. BCR-ABL has been shown to prevent apoptosis and induce resistance to cytostatic drugs and irradiation by modulation of DNA repair mechanisms, cell cycle checkpoints and Bcl-2 protein family members. Upon DNA damage BCR-ABL enhances homologous repair of DNA and prolongs activation of cell cycle checkpoints (G_2/M) providing more time for repair of otherwise lethal lesions [Knowles *et al* 2001, Advani *et al* 2002, Era *et al* 2002, Sattler *et al* 2001, Ferrando *et al* 2000, Anastasiadou *et al* 2003]. BCR-ABL and other protein kinases activate STAT5 that induces overexpression of RAD51 and RAD51 paralogue members (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3). They are mitotic human homologues of the E.coli RecA (RAD51 paralogues) and play an important role in homologous recombination repair of DNA double-strand breaks [Skorski *et al* 2002]. Moreover, by modification of anti-apoptotic members of the Bcl-2 family, like up-regulation of Bcl-x_L, BCR-ABL counteracts apoptotic signals induced by the DNA damage [Arlighaus *et al* 2002, Fernandez-Luna *et al* 2000]. The genetic and chromosomal instability of *BCR-ABL* positive cells is caused by BCR-ABL interference with DNA repair mechanisms members and deregulation of telomere length. The fusion proteins down-regulate DNA-dependent protein kinases, that are part of the non-homologous end joining repair system, and the basal transcription factor TFIIH, a multiprotein complex involved in the nucleotide excision DNA repair system. It is not currently understood why oncogenic protein tyrosine kinases, like BCR-ABL, down-regulate one DNA double-strand break repair system (DNA-protein kinases of non-homologous end joining) and up-regulate another (RAD51 paralogues of homologous recombination repair). The unrepaired or aberrantly repaired non-lethal DNA alterations resulting from spontaneous as well as drug-induced DNA damage can accumulate in the cells and lead to genomic instability with malignant progression of the disease. Oncogenic protein tyrosine kinases or their downstream mediators can also alter the telomere length. For example, DNA-dependent protein kinases, down-regulated by BCR-ABL, are critical for correct telomere capping. It was recently demonstrated that shorter telomeres are responsible for decreased chromosome stability and increased mutation rates [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Pane *et al* 2002, Anastasiadou *et al* 2003, Ravandi *et al* 2002, Scheijen *et al* 2002].

BCR-ABL also increases cell adhesive abnormalities (through activation of a Paxillin complex), which may play a role in the dissemination of malignant cells [Sattler *et al* 2000].

Increased levels of hnRNP (RNA-binding ribonucleoproteins involved in the regulation of mRNA processing, nucleocytoplasmic export and translation), like FUS, hnRNP A1 and hnRNP E2, have been found in leukaemic cells with *BCR-ABL* translocation, suggesting that an alteration of mRNA metabolism may contribute to the process of leukaemogenesis. hnRNP2 E2, induced by BCR-ABL protein, can inhibit the translation of *C/EBPα* mRNA, thus downregulating its expression at the translational level. *C/EBPα* dysregulation leads to the disruption of differentiation of the leukaemic cells [Perrotti *et al* 2002].

The BCR-ABL protein can be detected by monoclonal antibodies. These antibodies either recognise the breakpoint region, for example ER-FP1 against BCR-ABL p190 protein [van Denderen *et al* 1994] or bind to both, normal or rearranged BCR [Dhut *et al* 1988]. Another monoclonal antibody KOR-SA3544 has been described to react with a surface antigen sSA3544 (also NCA-50/90, CD66c) predominantly expressed on Ph-positive ALL cells. The antibody recognizes non-specific cross-reacting antigen NCA-50/90 (CD66c), one of the carcinoembryonic antigen (CEA)-related glycoproteins encoded by a member of the *CEA* gene family. However, this antigen is not specific for t(9;22) translocation and is also expressed in cytoplasm of various types of leukaemia cells [Sugita *et al* 1999].

In B-ALL, this translocation is an independent unfavourable prognostic factor, associated with a high risk of treatment failure and a very poor prognosis. Some studies suggest that patients with p210 BCR-ABL fusion protein may possibly have a better outcome than those with p190 BCR-ABL fusion [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 1999, Harrison *et al* 2000, 2001 and 2002].

The *ABL* gene can be rearranged also by several other translocations. In acute leukaemia (AML and ALL) t(9;12)(q34;p13) translocation joins the N-terminal portion of TEL to ABL, creating TEL-ABL fusion protein. A duplication of *ABL* in ALL has also been described [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, La Starza *et al* 2002].

2.1.2.2. E2A translocations

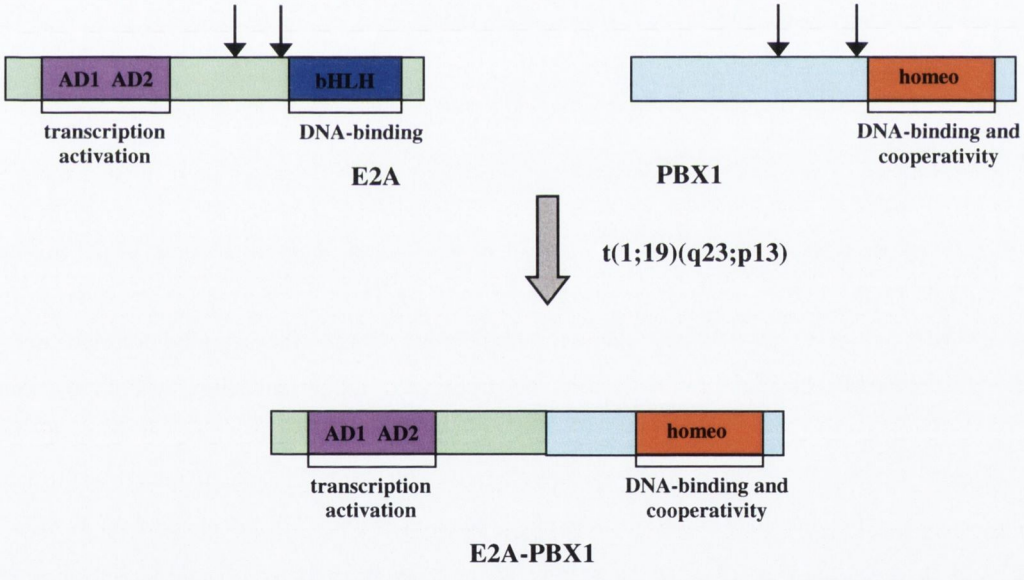
E2A-PBX1

The most common translocation affecting *E2A* is t(1;19)(q23;p13), where *E2A* gene on chromosome 19 is rearranged to *PBX1* on chromosome 1, forming a fusion gene *E2A-PBX1*. In 25 % of cases this translocation can be found in a balanced form, 75 % are unbalanced forms (-19, +der(19)t(1;19)(q23;p13), in which two normal chromosomes 1 are present). This translocation is associated with precursor B-ALL and can be found in about 5 - 15 % of childhood B-ALL, almost exclusively in pre-B-ALL cIgμ positive. It has also been reported sporadically in pro-B-ALL and common ALL, and in very rare cases of T-ALL and AML. An inversion of chromosome 19, inv(19)(p13q13) can also occur [Knowles 2001 *et al*, Degos *et al* 2000, van Dongen *et al* 1999, Harrison *et al* 2000, 2001 and 2002].

E2A gene encodes a transcription factor that is widely expressed in human tissues. It contains transcriptional activation domains (AD1 and AD2) at the N-terminal portion and a DNA-binding domain with the basic helix-loop-helix motif at the C-terminal part. *PBX1* is a homeobox gene, encoding a transcription factor that is ubiquitously expressed except in lymphoid lineages. It contains a DNA-binding

domain with the homeodomain motif, and the adjacent HOX-cooperativity domain, which is necessary for interactions with other homeodomain proteins (Figure 2.3.).

The *E2A-PBX1* fusion gene product consists of the N-terminal transactivation domain of E2A joined to the C-terminal DNA-binding homeodomain and HOX cooperativity domain of PBX1. This protein acts as a chimeric transcription factor and has been demonstrated to have oncogenic potential *in vitro* and *in vivo*. The oncogenicity of E2A-PBX1 appears to depend upon dimeric interactions of the fusion protein with other homeodomain proteins mediated by the HOX cooperativity region and on the conversion to a constitutive transcriptional activator mediated by the E2A transactivation domain. The ectopic expression of E2A-PBX1 in the B lineage, where PBX1 is not normally expressed, can lead to aberrant activation of tissue- and developmental-specific genes, normally regulated by the dimers formed between PBX1 and its partner homeodomain proteins. Genes that have been shown to be up-regulated by E2A-PBX1 fusion protein, include *G-CSF* (a growth factor), *EB1* (a tyrosine kinase signal transducer not normally expressed in B-cell precursors), *WNT16* (a member of the WNT gene family), and *angiogenin-3* (a member of the angiogenin gene family). All these observations strongly support the involvement of *E2A-PBX1* in the pathogenesis of B-ALL [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, van Dongen *et al* 1999, Harrison *et al* 2000, 2001 and 2002, Ferrando *et al* 2000].



AD1 and 2 - transcription activation domains
bHLH - basic helix-loop-helix
homeo - homeodomain

Figure 2.3. E2A-PBX1 translocation.

The E2A-PBX1 chimeric protein can be detected by a monoclonal antibody anti-E2A-PBX1 [Sang 1997]. Most of leukaemic cells with t(1;19) translocation express a typical immunophenotype, CD19, CD10 and CD9 positive, CD34 negative and CD20 weak positive or negative.

This translocation in B-ALL has been associated with worse prognosis, correlating with the presence of clinical high-risk features, such as elevated leukocyte count, high serum lactate dehydrogenase and CNS involvement. This negative prognostic value of *E2A-PBX1* rearrangement seems to be modified by intensive chemotherapy, which may reverse the unfavourable disease outcome. The unbalanced translocations in childhood B-ALL were shown to have a better outcome than the balanced form [Knowles 2001, Degos 2000, Harrison 2000, 2001 and 2002].

E2A-HLF

E2A can be also fused to the partner gene *HLF* in the chromosomal translocation t(17;19)(q22;p13), which occurs in 1 % of childhood precursor B-ALL. HLF (hepatic leukaemia factor) is a transcription factor that possesses a PAR (proline-rich and acidic amino acid-rich region) domain at its N-terminal portion and a basic leucine zipper domain, mediating the DNA binding and heterodimerization, at the C-terminus. HLF is normally expressed in liver but not in haematopoietic cells. The translocation generates a fusion protein composed of the N-terminal transactivation domain of E2A and the C-terminal leucine zipper domain of HLF. The transforming function of E2A-HLF depends on its ability to alter transcription of effector genes through a gain-of-function mechanism. It can induce genes that inhibit apoptosis. This translocation is associated with high leukocyte counts, frequent coagulation abnormalities and poor outcome [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, van Dongen *et al* 1999, Harrison *et al* 2002].

2.1.2.3. MLL in 11q23 translocations

Translocations affecting *MLL* (mixed lineage leukaemia) gene on chromosome 11q23 are associated with both ALL and AML. They are present in about 10 % of AML of different subtypes, most often being found in M4, M5, M1, and 5 % of childhood B-ALL cases. Rarely, they may be detected in T-ALL. In both ALL and AML, these translocations are very frequent in infant leukaemia, where they are detected in 50 - 70 % of ALL and around 30 % of AML cases. They are also present in many secondary leukaemias, particularly in relation to previous therapy with topoisomerase II inhibitors. In addition to translocations, *MLL* gene may be also mutated by a deletion of exon 11, associated with T-ALL. Tandem duplications of *MLL* have been observed in both *de novo* and secondary AML, ALL, as well as in normal haematopoietic cells [Knowles *et al* 2001, van Dongen *et al* 1999, Harrison *et al* 2000, 2001 and 2002, Hall *et al* 2001, Grimwade *et al* 2001].

MLL gene encodes a large scaffold protein, a putative transcription factor that shares homology with the Drosophila trithorax protein. It contains multiple DNA-binding structures: the N-terminal portion contains an AT-hook DNA-binding domain of high mobility group proteins that mediates binding to AT-rich sequences within the minor groove of DNA, and a cysteine-rich domain with homology to DNA methyltransferase with binding capacity to hemimethylated DNA. A zinc finger-like DNA-binding domain similar to trithorax is located centrally, and another domain with homology to trithorax (SET domain) that interacts with a component of the SNF/SWI complex, a chromatin remodelling system, is at the C-terminal portion. These domains may also allow MLL interactions with other DNA-binding

proteins. In addition to these, MLL protein contains an RNA binding region and a lysine-rich region with homology to histon H1. Normally, MLL regulates genes encoding homeobox transcription factors. It is thought to act by creating regions of active chromatin, thus permitting local gene transcription. Through its SET domain MLL also interacts with an anti-phosphatase called Sbf1 that acts as a positive regulator of kinase signalling pathways [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, Hall *et al* 2001, Grimwade *et al* 2001, Biondi *et al* 2000a, Armstrong *et al* 2002].

Several possible mechanisms may be implicated in the *MLL* gene rearrangement and are probably distinct in case of *de novo* and secondary AML. In *de novo* leukaemias the mechanism leading to *MLL* translocation may be a result of errors in DNA-repair mechanisms. Some *MLL* alterations are mediated by the VDJ recombinase and terminal nucleotidyl transferase mechanism, as a number of cases, particularly with t(9;11) and t(4;11), show presence of heptamer-nonamer-like sequences flaking the translocation breakpoint. Alu-repeat homologous recombination may be another mechanism involved. In secondary AML and also infant ALL, the inhibition of topoisomerase II by different agents leading to the accumulation of double-strand DNA breaks and consequent non-homologous recombinations are probably the principle cause. This is supported by the findings that in therapy-related AML and infant ALL the breakpoints are located in the telomeric half of the *MLL* breakpoint cluster region, where the high-affinity scaffold attachment regions (SAR; sites for binding of DNA to nuclear matrix thought to be involved in chromosomal rearrangements) as well as the topoisomerase II consensus binding sites are mapped. There is strong evidence that *MLL* rearrangements in many cases arise in the prenatal period. It also seems that interindividual (in case of infant ALL parental) pharmacogenetic-based differences in metabolism of topoisomerase II inhibiting agents, like variable activity of NAD(P)H quinone oxidoreductase (NQO1), an enzyme metabolising flavonoids and other quinine-based substrates, may be responsible for the different individual susceptibility to these agents. Dietary maternal exposure to bioflavonoids, pesticides and other chemicals have been linked to infant acute leukaemia with *MLL* rearrangement [Greaves *et al* 2003, Biondi *et al* 2000a].

MLL translocations are promiscuous, resulting in a fusion of the gene *MLL* with many alternative partner genes (more than 30 different translocations have been described and about 20 partner genes identified). The partner genes belong to a heterogeneous group and although some may share sequence homology, no common features have been found among them that would definitely explain the leukaemogenicity of the various fusion proteins they form with MLL. The partner genes, according to their function, are: transcription factors - *AF4* (4q21), *AF9* (9p21), *AF10* (10p12), *ENL* (19p13.3), *AFX* and *AF6q21* forkhead transcription factors; factors related to transcription - *CBF* (CREB-binding protein, transcriptional coactivator), *ELL* (RNA polymerase elongation factor); cytoplasmic signalling proteins - *EEN* (a protein with SH3 domain), *AbI-1* (ABL-interacting protein), *AF6* (Ras-binding protein, 6q27); septin family of cytoskeleton-associated GTPases - *MSF* (MLL septin-like fusion), *hCDCrel-1*, *AF17q25*; others - *AF1q*, *MEN*, *FEL*, *MLLT1*, *MLLT3* [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, van Dongen *et al* 1999, Harrison *et al* 2002, Biondi *et al* 2000a, Armstrong *et al* 2002].

The breakpoints in *MLL* span a certain defined genomic region, the breakpoints in the partner genes are more variable. The chimeric gene consistently found in malignant cells is the 5'-*MLL*-3'-partner fusion. The fusion protein contains the N-terminal portion of MLL, including the AT-hook domain, the

DNA methyltransferase domain and the zinc-finger-like domain fused to the partner protein domains [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, van Dongen *et al* 1999, Harrison *et al* 2002] (Figure 2.4.).

The mechanism by which MLL fusion proteins mediate the leukaemogenic process is not clear, but probably both the competition with the normal MLL protein function and creation of chimeric transcription factors with novel functions are involved. It is likely that MLL fusion proteins (all gene partners are fused to MLL in frame) interfere with the normal MLL function and regulation of chromatin structures at specific regions of the genome, which may be influenced by the different fusion partners. It is suggested that these fusion proteins induce malignant transformation through deregulation of genes by the gain-of-function mechanism, to which both of the partner fusion genes contribute. However, mice studies indicate that even truncated N-terminal part of MLL fused to any partner protein can be leukaemogenic, possibly as a result of the loss of the C-terminal domain with important functions in chromatin remodelling and regulation of gene expression. Thus in the transforming activity of MLL fusion proteins, new abnormal protein-protein interactions, altered interactions with chromatin-remodelling system, inhibition of apoptosis and probably

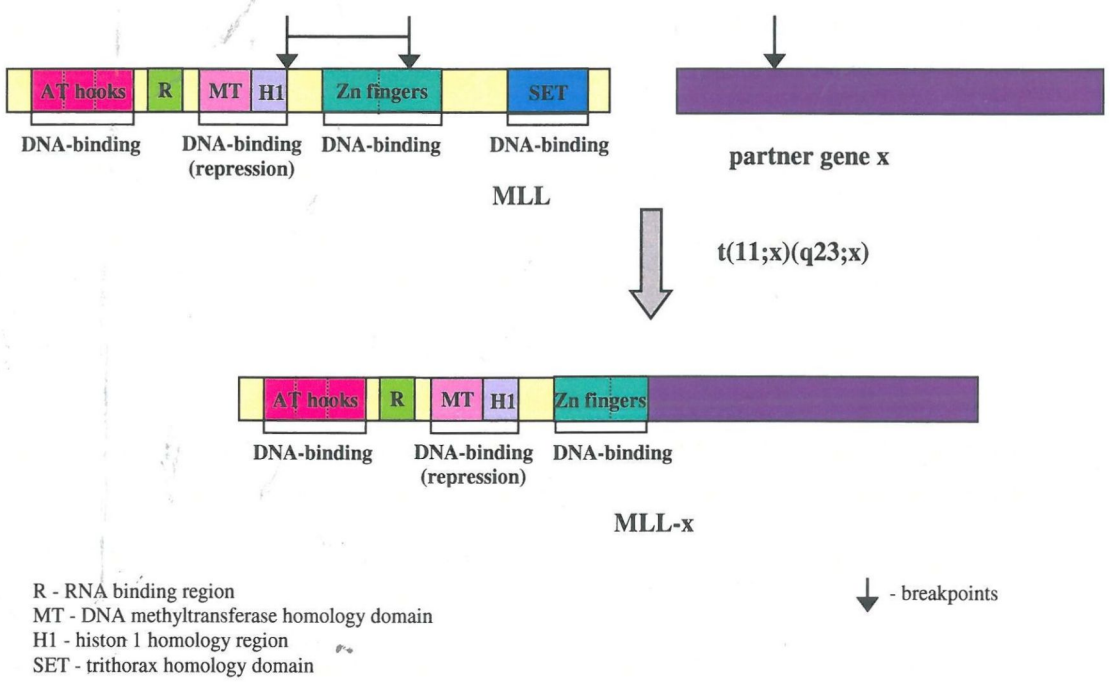


Figure 2.4. MLL translocations.

other mechanisms may be involved [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, Hall *et al* 2001, Grimwade *et al* 2001, Biondi *et al* 2000a, Armstrong *et al* 2002].

Leukaemic cells with *MLL* rearrangement frequently display mixed-lineage characteristics. The *MLL* gene rearrangement is also in many cases correlated with an expression of a cell-surface chondroitin

sulfate proteoglycan molecule called the rat homologue NG2 antigen (NG2). This molecule is recognised by the monoclonal antibody 7.1, which may be used for the immunological detection of cells containing the MLL translocations [Wuchter *et al* 2000].

The most common *MLL* translocation occurring in childhood B-ALL is the t(4;11)(q21;q23) translocation, resulting in the fusion of MLL with a transcription factor AF4, a not well defined protein with serine-proline-rich regions, on the chromosome 4. This translocation is associated predominantly with pro-B-ALL subtype. Cells with this *MLL-AF4* gene fusion express a characteristic phenotype, cCD79a and CD19 positive, CD10 and CD 24 negative, CD15 and CD65 positive (myeloid differentiation antigens co-expression), and also NG2 positive [Ferrando *et al* 2000, Knowles *et al* 2001, Harrison *et al* 2002, Biondi *et al* 2000a].

The most frequent translocations in AML are t(9;11)(q21;q23) with *MLL-AF9* rearrangement, t(11;19)(q23;p13.1) with *MLL-ELL* fusion and t(11;19)(q23;p13.3) with *MLL-ENL* fusion. The latter is also the most common MLL translocation observed in T-ALL, but still present only in rare cases [Kawagoe *et al* 2001, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Biondi *et al* 2000a].

The *MLL* rearrangements in ALL have been identified as an adverse prognostic factor associated with a dismal outcome, usually associated with other high-risk prognostic factors like a high leukocyte count, organomegaly and CNS involvement. The negative predictive value of *MLL* translocations in ALL is also influenced by age, with the worst prognosis for infant leukaemia. In AML, an association with worse prognosis has not been proved. Whether this is a result of interactions with mostly different partner fusion genes involved in ALL or AML cases respectively, needs to be clarified [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 1999, Harrison *et al* 2000, 2001 and 2002, Hall *et al* 2001, Grimwade *et al* 2001, Biondi *et al* 2000a].

2.1.2.4. RARA translocations

Several related reciprocal chromosomal translocations involve the *RARA* gene, a gene for the retinoic acid receptor α (RARα) on chromosome 17. These translocations play a crucial role in the pathogenesis and are very specific for acute promyelocytic leukaemia (APL, AML M3 subtype) including its morphological variants (microgranular and hyperbasophilic), which globally accounts for about 7 - 10 % of all AML [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 1999, Hall *et al* 2001, Grimwade *et al* 2001].

RARα is a ligand-activated transcription factor of the nuclear hormone receptor family. When linked with another member of this family, retinoic X receptor α (RXRα), this heterodimer can bind to DNA regulatory sequences. It also binds retinoic acid (RA) that activates the receptor. In the absence of RA, RARα/RXRα is bound to the corresponding DNA sequences forming a complex with nuclear co-repressors N-CoR/SMRT and Sin3A/B, and a histone deacetylase (HDAC). This complex induces deacetylation of lysine residues in histone proteins, resulting in transcriptional repression. Activation by RA causes a conformational change in the RARα subunit, resulting in dissociation of co-repressors from RARα/RXRα and their replacement by transcriptional activators like CBP and p300. These co-activators have histone acetylase activity and induce nucleosome unfolding, which induces transcription of

responding genes required for cellular differentiation and growth inhibition. RARα has a modular structure containing several functional domains, including a transactivation domain and DNA-binding domain with zinc finger motif at its N-terminal portion, and a ligand (RA)-binding domain and a RXRα heterodimerization domain at its C-terminal portion. The breakpoint affects *RARA* gene in the region between the transactivation and DNA-binding domains [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001].

In more than 99 % of APL cases, the t(15;17)(q22;q21) results in a reciprocal translocation of *RARA* to *PML* (promyelocytic leukaemia) gene on chromosome 15. This gene encodes a putative novel transcription factor, a serine/tyrosine phosphoprotein with predominant nuclear localization. It is associated with the nuclear matrix and forms macromolecular organelles called PML oncogenic domains (POD) or PML nuclear bodies, which are involved in proteasome-mediated degradation of ubiquitinated proteins [Mattsson *et al* 2001, Knowles *et al* 2001]. PML is a mediator of apoptotic pathways including Fas, TNF and INF signalling and acts as a promoter-specific transcription suppressor, proliferation and tumour suppressor. PML protein contains a unique cysteine and histidine-rich zinc finger-like domain called the RING finger, followed by two cysteine/histidine-rich regions known as B box domain. They are responsible for DNA binding. In the central part, PML contains a coiled-coil region mediating PML homo- and heterodimerization; serine/proline-rich region (S/P) is localised at its C-terminal portion. There are three possible breakpoint regions in *PML* gene, all within coiled-coil region to S/P rich region. PML-RARα fusion protein is formed by the RING and B box of PML, with (L, a long transcript) or without (S, a short transcript) the coiled-coil region, and RARα without its transactivation domain. Because of its structure, PML-RARα can interfere with the retionoic acid-mediated transactivation pathway and the normal function of the wild-type PML. The reciprocal fusion protein, consisting of the transactivation domain of RARα fused to the C-terminal portion of PML, has not been attributed with any definitive functions [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001] (Figure 2.5.).

In less that 1 % of APL cases, *RARA* translocates to *PLZF* (promyelocytic leukaemia zinc finger) gene on chromosome 11, t(11;17)(q23;q21), which codes for a transcription factor. Other *RARA* translocations, involving *NUMA1* (nuclear mitotic apparatus protein) gene, coding for an essential component for the function of a mitotic spindle on chromosome 11, t(11;17)(q13;q21), or *NPM* (nucleophosmin) gene, coding for an RNA-binding nucleolar phosphoprotein on chromosome 5, t(5;17)(q35;q21), are very rare [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001].

The result of the *RARA* translocations is the production of fusion proteins between RARα and the proteins encoded by the partner genes. All the fusion proteins are identical in their RARα-derived sequence, which retain the DNA-binding domain, the ligand (RA)-binding domain, as well as the domain for heterodimerization with RXRα. This is joined to the N-terminal portions of the partner proteins that all contain homodimerization domains.

RARA fusion genes are necessary but not sufficient for the leukaemogenic transformation. The oncogenicity of the chimeric fusion proteins appears to rely on the interference of retinoic acid-mediated transactivation in the RARα/RXRα pathway and on disruption of normal function of the RARα partner proteins. The fusion proteins can heterodimerize with RXRα, bind to DNA in forms of complexes with

co-repressors, they can bind RA but cannot activate the transcription. In the presence of physiological amounts of RA, they are unable to dissociate from the co-repressor complexes and act as potent dominant transcription repressors. The sensitivity of the fusion proteins complexes to RA determines the response to pharmacological doses of RA. High, pharmacologic doses of RA may induce a conformational change of PML-RARα and NPM-RARα fusion proteins leading to their dissociation from the complexes with co-repressors, reverting their inhibitory effect on gene transcription. In case of PLZF-RARα fusion protein, the co-repressor complex is directly bound by both parts of this protein, PLZF and RARα, the PLZF binding being unresponsive to high-dose RA. This determines the clinical features of APL where cases with PML-RARα or NPM-RARα translocations are all-trans-retinoic acid (ATRA) sensitive and have significantly better prognosis than those with PLZF-RARα translocations, which are resistant to ATRA therapy [Grimwade *et al* 2001 and 2002, Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Hall *et al* 2001]. The abnormal interactions of the fusion proteins with co-repressors leading to transcriptional silencing of RA-regulated genes are the principle mechanism involved in APL pathogenesis. A gene of a special importance inhibited by PML-RARα is *UBE1L* (ubiquitin-activating enzyme E1-like) gene. Transcription of this gene is normally activated by retinoic acid but repressed by PML-RARα protein. The enzyme, encoded by this gene, catalyses the proteasome-dependent degradation of various proteins, including PML-RARα. UBE1L is proposed to be a direct pharmacological target of ATRA effect that overcomes the oncogenic mechanism of PML-RARα by triggering its degradation and signalling apoptosis in ATRA-sensitive APL cells [Kitareewan *et al* 2002]. The anti-apoptotic effect of the RARα fusion proteins can also be blocked by arsenic trioxide (As_2O_3) [Grimwade *et al* 2002, Knowles *et al* 2001].

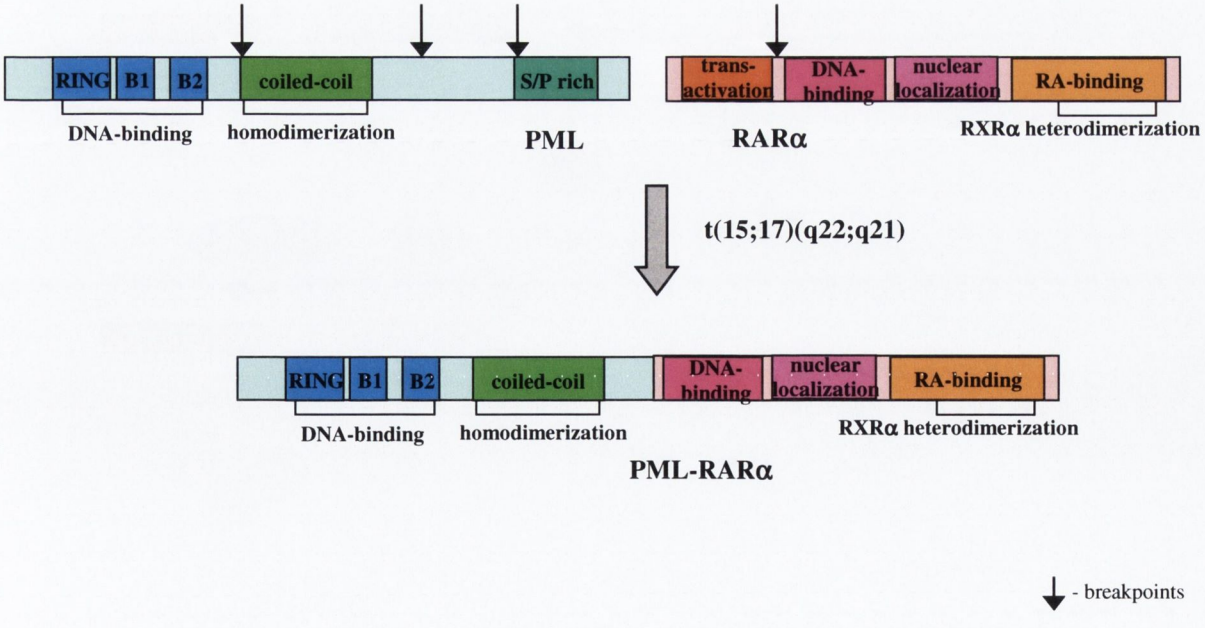


Figure 2.5. PML-RARA translocation.

The fusion proteins can also interfere with the normal functions of the native partner proteins through heterodimerization with them and inhibition of their normal function in a dominant-negative manner. PML-RARα fusion protein causes disruption of the PML nuclear bodies leading to apoptotic resistance. The alteration of the growth- and tumour-suppressing and apoptosis-inducing functions of PML significantly contribute to the leukaemogenic effect of *PML-RARα* translocation. This effect can be also reverted by treatment with ATRA (Figure 2.6.).

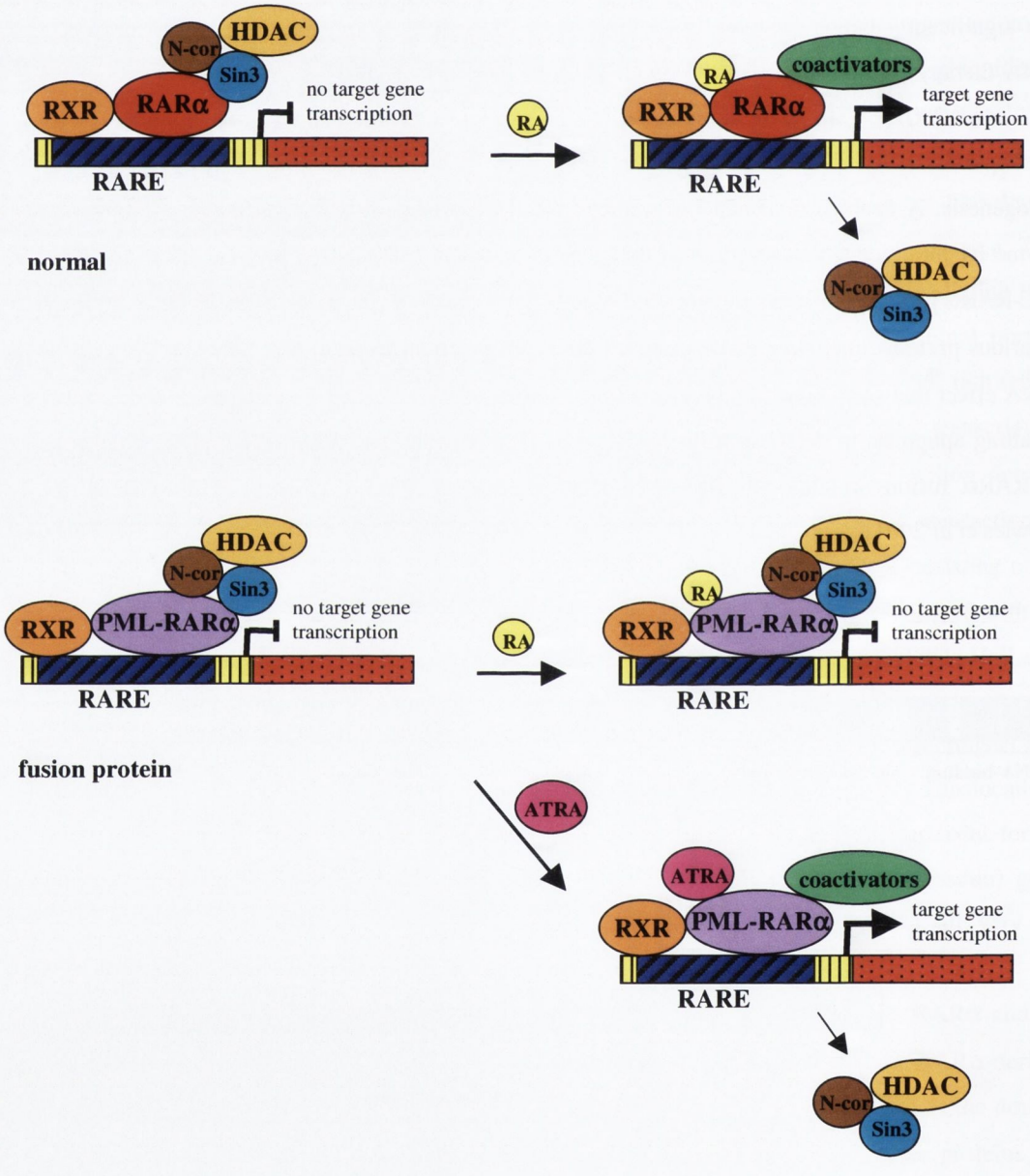


Figure 2.6. Function of normal RARα protein and fusion PML-RARα protein.

The reciprocal fusion proteins consist of the N-terminal transactivation domain of RARα and the C-terminal portion of the partner proteins. They can be found in many cases of APL but there is little known about their functions. Studies on mice suggest that they cannot induce the leukaemic process but possibly may contribute to its development in the presence of the counterpart fusion proteins [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001].

Cells with RARA translocations also show specific immunological features, CD13, CD33 and CD117 positive and CD34 and HL-DR negative. PML-RARα protein delocalisation towards the cytoplasm may be demonstrated by anti-PML antibodies [Orfao *et al* 1999, Degos *et al* 2000].

Translocations t(15;17) and t(5;17) are associated with a good response to ATRA and favourable clinical outcome, and represent thus a significant positive prognostic factor [Knowles *et al* 2001, Degos *et al* 2000, Hall *et al* 2001, Grimwade *et al* 2001].

2.1.2.5. AML1 translocations

AML1 is involved in many translocations, the two main ones in acute leukaemias are *AML1-ETO* and *TEL-AML1*. Apart from the translocations, *AML1* may be rearranged and deregulated by amplifications and point mutations.

AML1-ETO

The *AML1-ETO* translocation, t(8;21)(q22;q22) is present in approximately 10 - 15 % of AML, being more common in younger patients. In up to half of the cases, prenatal origin can be found. It is predominantly, but not exclusively, associated with M2 AML subtype; rare cases of AML M1, M4 and secondary AML have also been found to be t(8;21) positive. This translocation involves rearrangement of the *AML1* gene on chromosome 21 and the *ETO* gene on chromosome 8 [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 1999, Rubnitz *et al* 2002].

AML1 (*CBFA2*) encodes a widely expressed transcription factor that forms the α subunit of the heterodimeric core-binding factor (CBF, a heterodimer of AML1 and CBFβ), which is an essential regulator of haematopoiesis. It binds to the enhancer core sequence and influences transcription of many genes (eg. cytokines and growth factors, growth factor receptors, TCR, intracellular enzymes etc.). AML1 protein comes in various isoforms. AML1b, the longer isoform, contains a highly conserved runt homology domain at its N-terminal portion, which mediates specific DNA-binding and protein-protein interactions, including heterodimerization with CBFβ that increases AML1 DNA-binding affinity. The C-terminal end of AML1 protein contains a transactivation domain and nuclear-matrix-targeting signal that directs the protein to the appropriate nuclear matrix-associated foci. AML1a, the smaller isoform, lacks the C-terminal portion with the transactivating domain. AML1b and AML1a act antagonistically, AML1b as a transcriptional activator while AML1a as a repressor of transcription of the target genes.

ETO (eight twenty one; or cyclin D-related) gene product is a corepressor for PLZF protein and is not normally expressed in haematopoietic cells. It contains several proline/serine/threonine-rich (PST) domains. Its C-terminal region can interact with other co-repressors, such as SMRT and N-CoR. Like

AML1, it is also associated with nuclear matrix [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Roumier *et al* 2003, Perry *et al* 2002].

In case of the secondary therapy-related AML, the *AML1-ETO* translocation may be related to the presence of a consensus topoisomerase II cleavage motif in the region of AML1 gene, which is involved in the fusion to the ETO gene [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Perry *et al* 2002].

The t(8;21) translocation fuses the N-terminal part of AML1 containing the DNA-binding runt domain to near full-length ETO (Figure 2.7.). The fusion protein AML1-ETO can bind to DNA and dimerize with CBFβ, but the loss of AML1 transactivation domain and nuclear-matrix-targeting signal and fusion with ETO domains alters considerably the intranuclear targeting and transcriptional activity of this chimeric protein. AML1-ETO is misrouted by ETO component to alternate nuclear-matrix-associated foci, away from AML1 normal foci that support the appropriate gene expression. The substitution of the C-terminal portion of AML1 by the C-terminal part of ETO also generates an aberrant transcriptional regulator because of the ability of ETO to recruit the transcriptional co-repressors (Figure 2.8.). AML1-ETO fusion protein can therefore antagonise the normal AML1 protein function in a dominant-negative fashion. Both of these mechanisms may result in deregulation of AML1 effector genes, leading to abnormal proliferation of haematopoietic precursors and leukaemogenic development [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Roumier *et al* 2003, Perry *et al* 2002]. Cells with t(8;21) often express lymphoid markers, CD19, eventually CD56 [Degos 2000].

The t(8;21) translocation is associated with a relatively good prognosis in AML, particularly with a good response to certain therapeutic agents, notably cytarabine (cytosine arabinoside) [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 1999, Rubnitz *et al* 2002].

TEL-AML1

The t(12;21)(p12;q22) translocation, responsible for the *TEL-AML1* rearrangement, is observed in about 15 - 25 % of childhood precursor B-ALL, mostly in pre-B-ALL and common B-ALL subtypes. *TEL-AML*1 translocation is the most frequent genetic abnormality in childhood ALL but is rare in adults, and has not been described in infant leukaemia. Despite that, the majority of the cases may originate prenatally *in utero*. It is not detectable by conventional cytogenetics. This translocation is hypothesised to be initiated by staggered DNA double-strand breaks followed by incorrect DNA repair. Most cases (70 - 80 %) exhibit deletions in the non-rearranged homologous *TEL* allele, which is probably a postnatal event leading to the onset of the leukaemia [Knowles *et al* 2001, Degos *et al* 2000, Harrison *et al* 2000, 2001 and 2002, van Dongen *et al* 1999].

The TEL protein belongs to the ETS family of helix-loop-helix transcription factors and act as an inhibitor of transcription. It contains an N-terminal "pointed" helix-loop-helix domain that has transcriptional repression activity, and the ETS domain at its C-terminus, that mediates specific DNA binding. TEL is widely expresses in haematopoietic and non-haematopoietic tissues. AML1 is a transcription factor that constitutes the α subunit of the heterodimeric CBF.

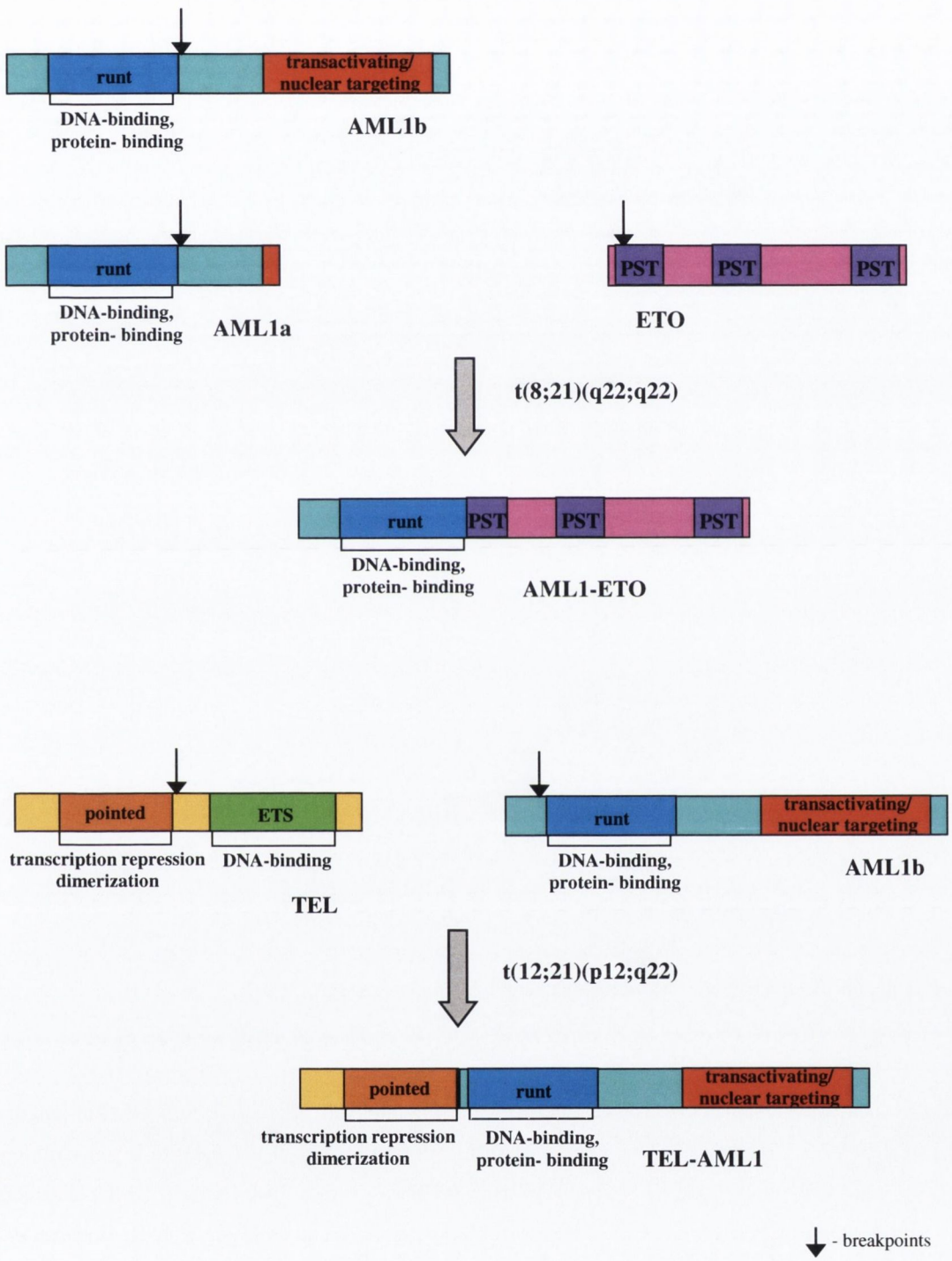


Figure 2.7. AML1 translocations.

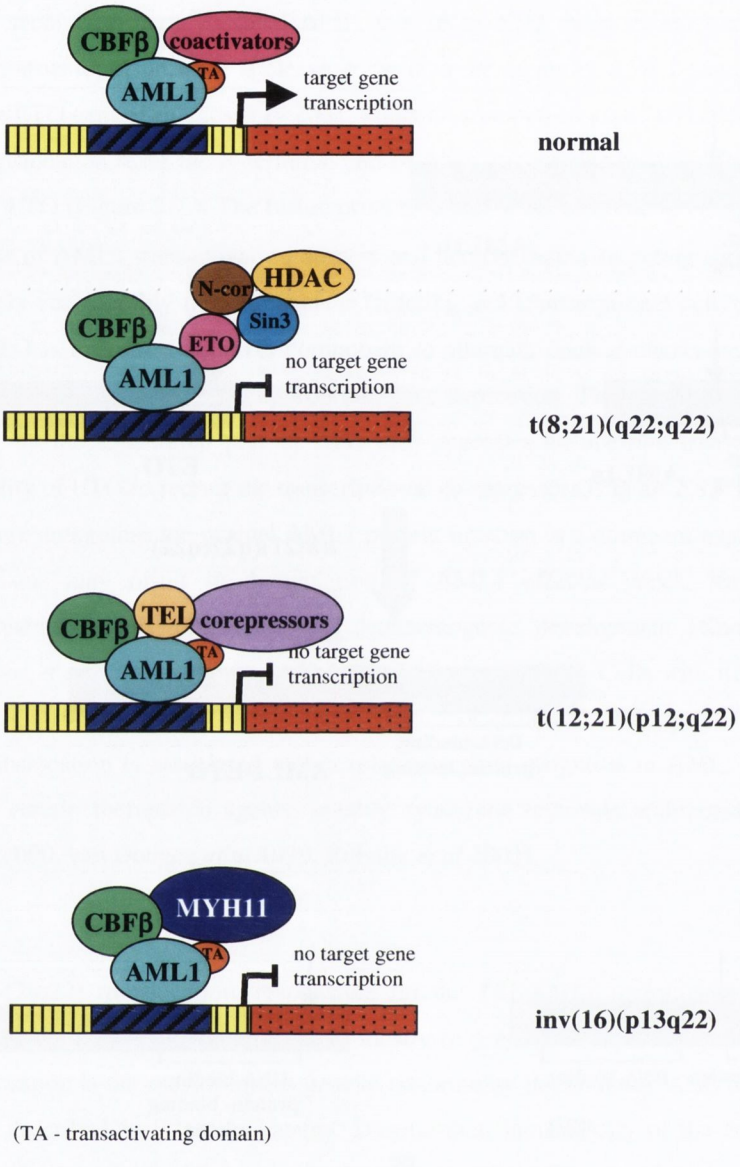


Figure 2.8. AML1/CBFβ function and its alteration by gene rearrangements.

The translocation result in the fusion of the N-terminal pointed domain of TEL to nearly full-length of AML1 (Figure 2.7.). This fusion converts AML1 from a transcriptional activator to a transcriptional repressor and may contribute to leukaemogenesis by the specific inhibition of AML1-dependent activation of its normal target genes [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, van Dongen *et al* 1999, Perry *et al* 2002, Ferrando *et al* 2000] (Figure 2.8.).

Immunophenotyping of cells with t(12;21) shows co-expression of myeloid markers. Cells with this translocation also do not have major numerical chromosomal abnormalities, and are not hyperdiploid [Degos *et al* 2000].

This translocation has correlated with an excellent outcome and positive clinical prognostic features, like low leukocyte count. More recent findings however suggest that it could be associated with late relapse [Knowles *et al* 2001, Degos *et al* 2000, Harrison *et al* 2000, 2001 and 2002, van Dongen *et al* 1999].

Several other translocations involving *TEL* may be detected in ALL. In translocation t(7;12)(q36;p13) *TEL* is fused to *HOXB9*, a homeobox gene on chromosome 7. t(9;12)(q11;p13) juxtaposes *TEL* to *PAX5*, a gene encoding a transcription factor with paired box domain and zinc finger motif on chromosome 9. t(9;12)(q34;p13) generating the fusion gene *TEL-ABL* have been mentioned in BCR-ABL translocation. t(9;12)(p24;p13) translocation disrupts *TEL* to *JAK2* on chromosome 9, resulting in constitutive activation of this tyrosine kinase [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2000, 2001 and 2002].

Other translocations involving AML1

Further translocations involving *AML1* include t(3;21)(q26.2;q22) with *AML1* fusion to three different genes, *EVI1*, *EAP* or *MDS1*. These translocations are detected rarely in *de novo* AML, but often found in therapy-related AML. *AML1-EAP* gene fusion results in an out-of-frame EAP product, and thus encodes a truncated AML1 with a repressor function. *MDS1* is a poorly defined gene that may be present as *MDS1* transcript alone or *MDS1-EVI1* fusion transcript. EVI1 is a nuclear DNA-binding protein with multiple zinc finger motifs at the N-terminal part and an acidic domain at the C-terminus that binds to a sequence that includes the binding site for GATA transcriptional activators. It is not normally expressed in the majority of haematopoietic cells but is overexpressed as a result of gene alterations and then inhibits normal GATA-mediated transcriptional activation. Translocation t(16;21)(q24;q22) fuses *AML1* to *MTG16* gene and occurs very rarely in AML, usually therapy-related cases. Other chromosomal bands have been shown to be rearranged to the *AML1* locus, including 1p36, 5q13, 14q22, 15q22, 17q11 and 20p13 [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Grimwade *et al* 2001, van Dongen *et al* 1999].

EVI1 gene is also altered in other abnormalities affecting 3q21, like inv(3)(q21q26) or t(3;3)(q21;q26), that are found infrequently in AML [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Grimwade *et al* 2001].

2.1.2.6. CBFB alterations

The core-binding factor βa (CBFβ) gene (*CBFB*) on chromosome 16q is disrupted by a pericentric inversion inv(16)(p13q22) and its variants, translocation t(16;16)(p13;q22) or deletion del(16)(q22). These aberrations are detected in 5 - 10 % of AML involving all M4Eo subtypes and rarely in other AML subtypes (all except M3) [Knowles *et al* 2001, Degos *et al* 2000, Grimwade *et al* 2001, van Dongen *et al* 1999].

The *CBFB* gene on chromosome 16q encodes a ubiquitously expressed heterodimeric transcription factor CBFβ that forms, as a β subunit, an active dimer with AML1 (CBFα) transcription factor, the α subunit. The N-terminal portion of CBFβ contains AML1-interactive domain that realises the binding

with AML1. AML1 itself can bind to the regulatory DNA sequences but with a low affinity. CBFβ cannot bind the DNA directly but in form of the heterodimer enhances the binding of AML1 to DNA [Knowles *et al* 2001, Degos *et al* 2000, Downing *et al* 2001].

In 16q abnormalities, *CBFB* gene is rearranged to the smooth muscle myosin heavy chain gene (*MYH11*) on chromosome 16p, which contains rod domain at its C-terminal part. Two different breakpoint regions in *CBFB*, near the 3' end of the coding region, and heterogeneity of breakpoint regions (seven have been identified, all within the conserved tail region) in *MYH11* give rise to ten different fusion gene transcripts. The results of these rearrangements are CBFβ-MYH11 fusion proteins that retain the CBFβ heterodimerization AML1-interactive domain and myosin multimerization rod domain and exhibit transforming properties (Figure 2.8. and 2.9.). They directly repress AML1 mediated transcriptional activation, and interfere with and inhibit normal transcriptional regulation by the AML1/CBFβ dimers, probably by their sequestration into functionally inactive complexes within the nucleus [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Grimwade *et al* 2001, Lorsbach *et al* 2001, Downing *et al* 2001, Kundu *et al* 2001].

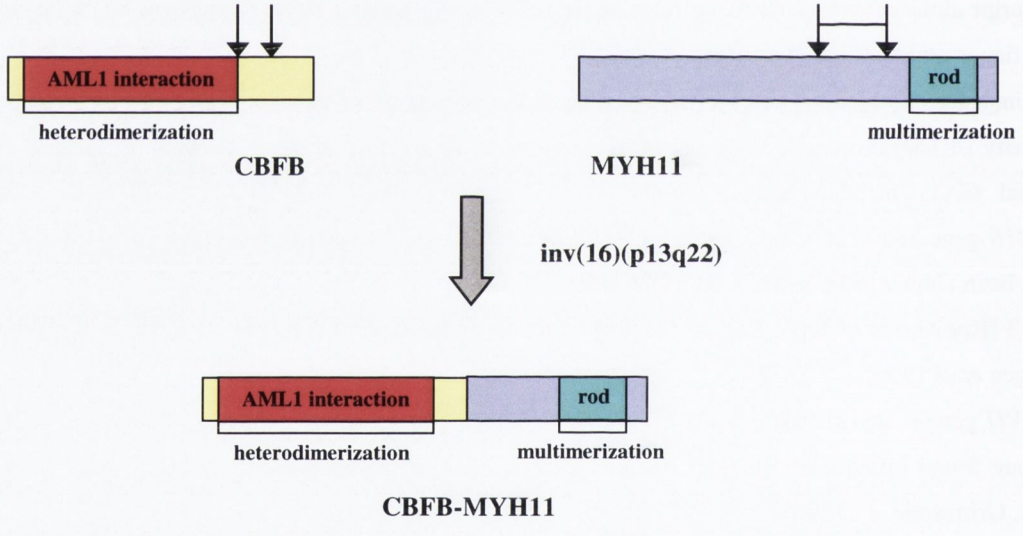


Figure 2.9. CBFB-MYH11 translocation.

The prognostic significance of inv(16) in AML remains controversial. Similar to *AML1-ETO* translocation, it is usually associated with remarkable sensitivity to high-dose cytarabine [Knowles *et al* 2001, Degos *et al* 2000, Grimwade *et al* 2001].

In certain cases of *CBFB-MYH11* alteration, an additional deletion of 16p, proximal to the breakpoint and encompassing the multi-resistance protein (MRP)-coding region may take place but no clinical significance of this deletion has been shown [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Lorsbach *et al* 2001].

2.1.2.7. TAL1 translocations

TAL1 (*SCL*) gene, located on chromosome 1p32, is the most frequently rearranged gene in precursor T-ALL and can be identified in up to 30 % of T-ALL cases. It encodes a transcription factor with the basic helix-loop-helix DNA-binding motif (bHLH), which shows a marked homology to two other helix-loop-helix transcription factors involved in chromosomal translocations in T-ALL, LYL1 and TAL2. In a form of a heterodimer with the E-box bHLH transcription factors (ubiquitous members of E2A family) TAL1 binds to E-box enhancers and is supposed to function as a transcriptional regulator that influences cell-type determination in haematopoiesis and is essential for the development of all haematopoietic lineages. In normal T cells, TAL1 is not expressed [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2000, 2001 and 2002, van Dongen *et al* 1999, Ferrando *et al* 2000].

In less than 5 % of T-ALL cases, *TAL1* is altered by the translocation t(1;14)(p32-33;q11), where almost entire *TAL1* coding sequence is juxtaposed to the *TCRA/D* gene coding for TCRα/δ on chromosome 14, resulting in *TAL1-TCRA/D* fusion gene formation [Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, van Dongen *et al* 1999].

The most common *TAL1* alteration, found in about 10 - 25 % T-ALL cases, are cytogenetically undetectable site-specific deletions of approximately 90 kb that affects the 5' portion of *TAL1*. These microdeletions juxtapose the coding exons of *TAL1* to the non-coding exon of *SIL* (SCL interrupting locus). The *SIL* gene is located centromeric to *TAL1*. Its first two exons (1a and 1b) are non-coding. The function of *SIL* is not well-defined but it constitutes a member of the immediate-early gene family and is actively transcribed during T-cell development. The *SIL-TAL1* rearrangement results in a formation of a fusion gene that contains the 5' part of *SIL*, including its promoter, and entire protein-coding sequences of *TAL1*. The protein product of the *SIL-TAL1* fusion thus contains a full-length *TAL1* transcript. The breakpoints are located to several donor-deletion sites in *SIL* and acceptor-deletion sites in *TAL1*, where they are clustered at the site of heptamer-like recombinase recognition signal sequences, giving rise to three different *SIL-TAL1* transcripts. Based on the homology between the breakpoint junction sequences and the recombination signal sequence of Ig/TCR, the *SIL-TAL1* rearrangement is thought to be mediated by aberrant VDJ recombinase function and represent an illegitimate V(D)J recombination. These site-specific microdeletions are restricted to T cells of the TCRα/β lineage [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2000, 2001 and 2002, Ferrando *et al* 2000].

Both these alterations lead to the ectopic expression of *TAL1* in T cells governed by the regulatory elements of the fusion partner genes, *TCRD* and *SIL* respectively. The oncogenic function of TAL1 has been demonstrated *in vivo* and *in vitro* but the mechanism is unknown. It is proposed that TAL1 may compete with other tissue-specific HLH proteins for binding to common E-box HLH proteins, thereby interfering with the formation of heterodimeric transcription factors that drive cellular differentiation [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, Ferrando *et al* 2000].

2.1.2.8. MYC translocations

The *MYC* gene on chromosome 8q24 encodes c-MYC, a transcription factor critically involved in cell proliferation. This gene is rearranged in chromosomal translocations bringing *MYC* to actively transcribed immunoglobulin or T cell receptor gene loci.

The t(8;14)(q24;q32-3) translocates *MYC* to Ig heavy chain locus (IgH) on chromosome 14 (the orientation of the genes is 5' to 5', head to head); in t(2;8)(q12;q24) and t(8;22)(q24;q11) *MYC* is rearranged to Ig light chain loci, Igλ on chromosome 2 and Igκ on chromosome 22, respectively (the orientation of the genes in this case is 5' to 3', head to tail). These translocations are associated with non-Hodgkin lymphomas of Burkitt's type and with the rare B-ALL L3, where their presence is obligatory for the diagnosis. The t(8;14)(q24;q21) translocation may occur in about 2 % of T-ALL. In this alteration, the *TCRA* gene at 14 chromosome is juxtaposed to *MYC*.

The translocations may be initiated by DNA double-strand breaks arising during immunoglobulin gene rearrangements (VDJ rearrangements and somatic hypermutations) and result from errors of the VDJ recombinant machinery. The MYC breakpoints are highly variable, in t(8;14) typically in the first exon or intron, in t(2;8) and t(8;22) more than 20 kb upstream, leaving the structure of *MYC* intact. In IgH, the breakpoints are located within the switch (S) region or joining (J) region, occasionally in variable (V) or constant (C) regions. In all of the alterations the entire coding sequences of *MYC* (exon 2 and 3) are joined with the regulatory elements of the actively transcribed genes, immunoglobulins or T cell receptor, leading to transcriptional deregulation of *MYC* expression. The overexpressed *MYC* then stimulates several crucial pathways, involved in proliferation, differentiation and inhibition of apoptosis [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2000 and 2002, Boxer *et al* 2001, Hoffman *et al* 2002].

These translocations are associated with a very aggressive course of B-ALL but may be relatively successfully treated according to the protocols for Burkitt's and Burkitt's-like lymphomas.

2.1.2.9. Other translocations

DEK-CAN translocation

The translocation t(6;9)(p23;q34) is detected at a low frequency in AML (about 1 % cases). It is mostly found in M1, M2 and M4 subtypes, and appears to be associated with TdT-positivity and unfavourable prognosis. In this translocation, the 5' part of the gene *DEK* on chromosome 6 containing almost the entire coding sequence is fused to the 3' part of the gene *CAN* on chromosome 9. DEK is a nuclear protein, posses an activation domain at its N-portion and functions as a site-specific DNA-binding protein. It has been demonstrated to be an autoantigen in a subtype of juvenile rheumatoid arthritis and also a molecule binding to the human immunodeficiency virus (HIV) enhancer, suggesting it may function as a transcriptional regulator. CAN is a nucleoporin associated with the nuclear pore complex of the nuclear envelope and plays a crucial role in nucleocytoplasmic transport process.

The breakpoints at *DEK* and *CAN* occur at specific introns. This results in the generation of an invariable fusion transcript that encodes the DEK-CAN fusion protein. Its function and the significance of

DEK-CAN relocation to the nucleus in relation to its oncogenic properties remain to be determined. It is probable that alteration of the transcriptional activity of DEK protein and alteration of the nucleoporin structure with consequent changes in transport of RNA and proteins across the nuclear membrane can contribute to the malignant transformation [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Grimwade *et al* 2001].

TTG translocations

TTG1 and *2* (also *LMO1* and *2*) are members of the rhombotin gene family. They encode non-DNA-binding transcriptional regulators that possess the cystein-rich LIM domains, which mediate protein-protein interactions. *TTG1* is normally expressed in neural lineage cells but not in T cells. *TTG2* is strongly expressed in haematopoietic progenitors of the myeloid and erythroid lineages but at very low levels in T lymphoid precursors. TTG2 can interact with TAL and GATA-1 in an erythroid transcriptional complex, and plays a central role in early haematopoiesis, probably at the stage of pluripotent stem cells.

TTG1 is affected in t(11;14)(p15;q11) translocation, which is a rare chromosomal abnormality. *TTG2* is a target of translocations t(11;14)(p13;q11) that is found in 5 – 10 % of T-ALL. The translocations result in an association of *TTG1* or *2* genes on chromosome 11 with *TCRA/D* gene on chromosome 14, leading to deregulated or ectopic expression of *TTG1* or *2* with leukaemogenic potential. This may depend on disturbing T-cell differentiation through gene deregulation [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Grimwade *et al* 2001].

MOZ-CBP translocation

These genes are affected in a rare t(8;16)(p11;p13) translocation, that can be detected in about 0.5 % of AML, mostly of the M5 subtype. The gene on chromosome 16, encoding CREB (cAMP response binding protein)-binding protein (CBP), a transcriptional adaptor and co-activator protein, is disrupted by this translocation to a novel gene *MOZ* (monocytic leukaemia zinc finger) on chromosome 8, encoding a zinc finger protein with acetyltransferase activity. The fusion protein MOZ-CBP combines MOZ DNA-binding motifs with an almost intact transcriptional co-activator CBP and displays a transforming potential. This aberration is associated with particular clinical features, including frequent CNS involvement and coagulation defects [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Grimwade *et al* 2001].

Translocations specific for childhood acute leukaemia

Certain translocations are unique for childhood acute leukaemia, mainly AML, and are not present in adults. The t(1;22)(p13;q13) translocation occurs in less than 1 % of childhood AML but is found in majority of infants with M7 AML, usually as the sole abnormality. The breakpoints, recently characterised, involve two new genes, the megakaryocytic leukaemia gene (*MAL*) on chromosome 22 and the one twenty-two gene (*OTT*) on chromosome 1. The resulting OTT-MAL fusion protein is predicted to have a function in regulating chromatin organisation and *HOX* gene expression [Hall *et al* 2001, Grimwade *et al* 2001, Ballerini *et al* 2003].

Other examples are translocations involving nucleoporin *NUP98*, like t(5;11)(q35;p15.5) with *NUP98-NSD1* rearrangement, and translocations involving homeobox genes, like t(7;12)(q36;p13) with *HLXB9-TEL* rearrangement [Hall *et al* 2001, Grimwade *et al* 2001].

2.1.2.10. Homeobox genes alterations

Homeobox genes are a group of transcription factors that regulate the formation pattern and tissue identity during embryogenesis, and are also involved in regulation of haematopoietic cells proliferation and differentiation, particularly at the early stages of haematopoiesis. They are characterised by the presence of a conserved DNA-binding domain, the homeobox or homeodomain with the helix-turn-helix motifs, consisting of 4 helices. A connective hinge region (IYPWM) joins the homeobox with the variable domain that provides the binding specificity of the different homeoproteins and mediates the cooperative interactions with other regulatory proteins. The aminoterminal region (MSSLYY-N) may, at least in some homeobox genes, act as a transcriptional repressor, the carboxyterminal domain (the acidic tail) is supposed to function as a transcription activation domain [van Oostveen *et al* 1999, Owens *et al* 2002, Buske *et al* 2000, Thorsteinsdottir *et al* 2001].

Homeobox genes are divided into 2 main groups: class I and II. Class I, clustered homeobox genes constitute the *HOX* family containing 39 genes highly homologous (more than 80 %) to *Antennapedia* gene of Drosophila. They are organised in 4 clusters, *HOXA*, *B*, *C* and *D*, located on chromosomes 7, 17, 12 and 2, respectively. Genes of each cluster show positional arrangement (3' – 5') on the chromosomes, which correlates with their expression in body parts (anterior - posterior) and stages of embryogenesis or haematopoiesis (early - late). *HOX* genes belonging to different clusters but with highly homologous homeodomains can be aligned into 13 paralogous groups (*HOX1 - 13*) (Figure 2.10.). Class II, divergent homeobox genes, non-HOX genes, are more diverged (less than 50 % homology to the *Antennapedia* gene) and dispersed throughout the genome. Apart from the homeobox they possess additional conserved sequences, according to which they are grouped into several families: *PAX* (paired box; includes several *PAX* genes), *POU* (POU box; includes genes *OCT* and *PIT*), *TALE* (three-amino-acid loop extension, includes genes *PBX*, *MEIS* and *PREP/KNOX*), and many others (*HOX11* and *HOX11L2*, *HLX*, *DLX*, *HEX*, *PMX*, *LHX*, *GBX* and *CDX*). Some of them, for example *TALE* genes, function as cofactors of *HOX* genes [van Oostveen *et al* 1999, Owens *et al* 2002, Buske *et al* 2000, Thorsteinsdottir *et al* 2001, Thompson *et al* 2003]. In the haematopoietic system, only genes of *HOX A*, *B* and *C* clusters and certain genes of class II are normally expressed and this expression exhibits stage- and lineage-specific patterns. The expression of *HOXA* genes is generally higher than that of *HOXB* and *C*. Also, some *HOX* genes are expressed in all lineages but most are lineage-restricted [Magli *et al* 1997, Chiba *et al* 1998, Shimamoto *et al* 1998].

Homeoproteins regulate the transcription of many target genes encoding other transcription factors as well as homeobox genes themselves (auto- and cross-regulation), adhesion molecules and other proteins. In most cases they act in a form of complexes with other regulatory proteins and cofactors and may influence the transcription of the target genes in both ways, positively or negatively. In haematopoiesis, homeobox genes control the development of blood cells, promote their proliferation and block terminal

differentiation. The expression of homeobox genes is regulated by several principal and other additional factors. Apart from homeobox themselves, MLL transcription factor is the main regulator of homeobox genes, strongly activating their expression. Retinoic acid controls (activates or represses) homeobox gene expression via RARE (retinoic acid response elements) enhancers, transcription factors homologous to *Polycomb* gene in Drosophila repress homeobox gene expression [Magli *et al* 1997, Chiba *et al* 1998, Shimamoto *et al* 1998, van Oostveen *et al* 1999, Owens *et al* 2002, Buske *et al* 2000, Thorsteinsdottir *et al* 2001, Thompson *et al* 2003].

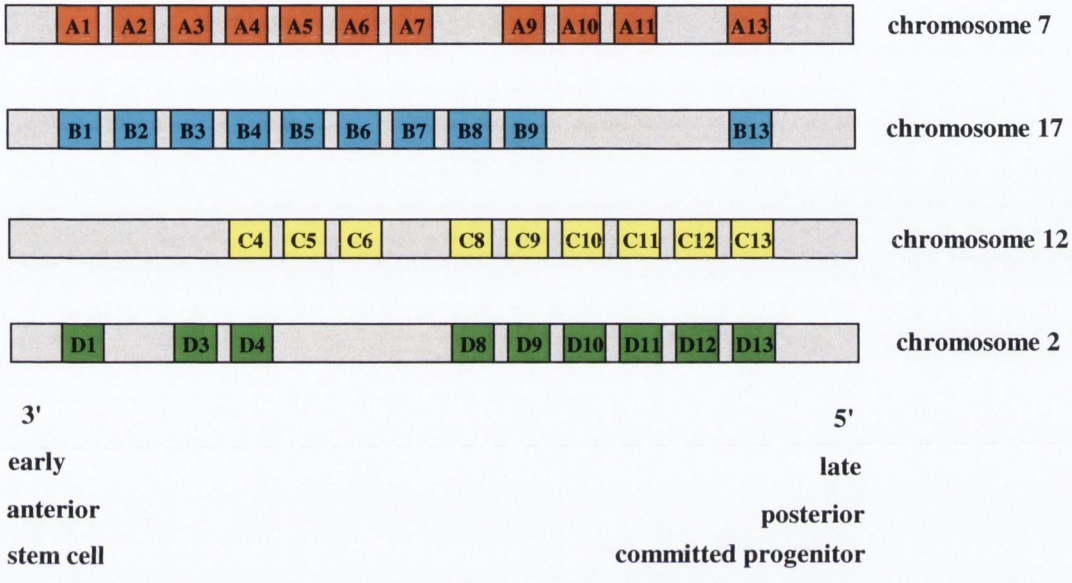


Figure 2.10. HOX genes arrangement and expression.

Alterations of homeobox genes show a significant leukaemogenic potential. Usually, a secondary mutational event is required for the full progression to malignancy, though in some cases the alteration of a single homeobox gene was shown to have oncogenic transforming capacity. Oncogenetic evens affecting homeobox genes lead to their dysregulated expression or creation of a fusion protein, which acts as a transcription factor with abnormal properties [van Oostveen *et al* 1999, Owens *et al* 2002, Buske *et al* 2000, Thorsteinsdottir *et al* 2001, Thompson *et al* 2003].

The translocation t(1;19)(q23;p13) with *PBX1-E2A* rearrangement has already been mentioned, as well as translocations that juxtapose a homeobox gene to *TEL*, t(7;12)(q36;p13) *TEL-HOXB9*, and t(9;12)(q11;p13) *TEL-PAX5*. Another translocation, rarely detected in AML, is t(12;13)(p13;q12) that rearranges *TEL* on chromosome 12 to *CDX2* homeobox gene on chromosome 13. Abnormal activity of CDX2, which is a regulator of class I homeobox genes, is hypothesised to be responsible for the dysregulated malignant proliferation [Chase *et al* 1999, Shimamoto *et al* 1998, Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2000 and 2002].

HOX11 gene is involved in the translocations t(10;14)(q24;q11) and t(7;10)(q35;q24), which are observed in about 5 % of precursor T-ALL. *HOX11* on chromosome 10 codes for a transcription factor of the class II homeobox family, controlling expression of developmentally important genes related to the

genesis of the spleen. In the t(10;14) translocation, the *HOX11* gene on chromosome 10 is juxtaposed to the *TCRD* gene on chromosome 14, in t(7;10) it is rearranged to *TCRB* on chromosome 7. Both translocations result in an ectopic overexpression of *HOX11* in T-precursors governed by the active regulatory elements of TCR genes. The mechanism of leukaemogenicity of HOX11 is not quite understood but may depend on its ability to promote cell survival or to abrogate the G_2/M cell cycle checkpoint. HOX11 interactions with downstream genes like *CTF1* (CCAAT-box-binding transcription factor 1) or *WT1* (Wilms' tumour gene 1) may also be involved. These translocations are associated with a more favourable outcome in T-ALL [Hawley *et al* 1997, Allen *et al* 2000, Shimamoto *et al* 1998, Knowles *et al* 2001, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2000 and 2002].

The translocation t(5;14)(q35;q32) was recently identified to be a major abnormality in childhood T-ALL, being found in 20 – 35 % of T-ALL cases. It affects an orphan class II homeobox gene *HOX11L2* (Hox11Like2), mapped close to the chromosome 5 breakpoints. This gene is transcriptionally dysregulated as a result of this translocation, probably through the influence of transcriptional regulation elements of CTIP2, localised in the vicinity of the chromosome 14 breakpoints and highly expressed during normal T-cell differentiation. This translocation has been shown to be associated with poor prognosis [Ballerini *et al* 2002, Shimamoto *et al* 1998, Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2000 and 2002, van der Velden *et al* 2003].

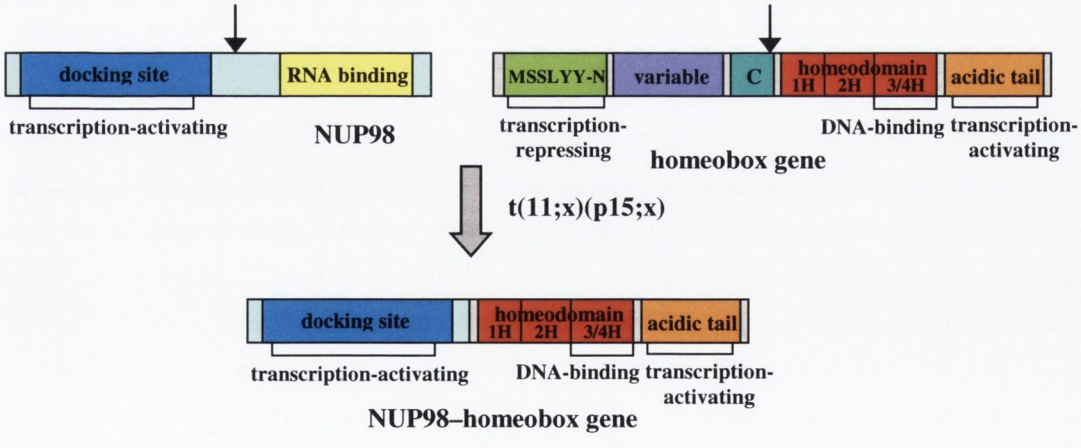
Several translocations that fuse a homeobox gene with nucleoporin gene *NUP98* have been detected in AML, predominantly in M2 - 4 subtypes and therapy-related AML. *NUP98*, located on chromosome 11p15, encodes a ubiquitously expressed protein that contains a FG (phenylalanine-glycine)-rich docking site at its N-terminal region and a RNA-binding domain at its C-terminal region. The N-terminal part exhibits strong transcription activating functions. The breakpoints occur in between the two main functional parts of *NUP98*. It is suggested that topoisomerase II inhibitors may be responsible for a proportion of these translocations and topoisomerase II-sensitive sequences have been found around the breakpoints of *NUP98*. In the translocation t(7;11)(p15;p15) *NUP98* is rearranged to *HOXA9*, *HOXA11* or *HOXA13* genes on chromosome 7. The breakpoints of *HOX* genes are located within introns of *HOXA9*, *HOXA11* and *HOXA13* [Fujino *et al* 2002]. It was also demonstrated that a single translocation may produce more than one *NUP98-HOXA* fusion protein, presumably because of alternative splicing. In the translocation t(2;11)(q35;p15) *NUP98* is joined to *HOXD13*, in the translocation t(11;12)(p15;q13) *NUP98* is rearranged to *HOXC13* [La Starza *et al* 2003]. The translocation t(1;11)(q23;p13) brings *NUP98* to *PMX1*, a class II homeobox gene related to paired box family but lacking the paired domain and containing the homeodomain only [Nakamura *et al* 1999]. In all these translocations, the N-terminal part of NUP98 containing NUP98 promoter and the transcription activation domain is rearranged to the C-terminal part of homeobox genes containing the DNA-binding homeodomain (Figure 2.11.). Both the homeobox- and the NUP98-derived portions are essential for the leukaemogenic properties of the fusion gene products, which show constitutive expression and altered transcriptional activity. FG repeats of NUP98 and sequences of certain *HOX* genes may interact with CREB-binding protein (*CBP/p300*), an important transcriptional co-regulator with inherent histone acetyltransferase acitivity, which can mediate the transforming effects of the *NUP98*-homeobox gene fusion proteins. The leukaemogenic activity may be potentiated by the loss of N-terminal transcription-repressing domain of the fused HOX protein.

Abnormal binding of HOX cofactors, PBX and MEIS, contribute to the pathological activity of NUP98-HOXA9 fusion protein [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Nakamura *et al* 1999, Fujino *et al* 2002, La Starza *et al* 2003].

In a similar way *NUP98* is disrupted to the putative RNA helicase gene *DDX10* on chromosome 11 in the inv(11)(p15;q22), and to a novel gene *NSD1* in the translocation t(5;11)(q35;p15.5), both found in rare cases of childhood AML [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001].

Dysregulated expression of a number of homeobox genes has been demonstrated in many cases of leukaemia and may contribute to the leukaemogenic process. Apart from specific gene rearrangements, this may be a result of alterations affecting factors that control homeobox gene expression. A defect of MLL transcription factor (including *MLL* translocations), retinoic acid influence (including *PML-RARA* translocation) or unbalances in the concentration of the cofactor proteins PBX or MEIS can significantly change the expression of homeobox genes. The pattern of homeobox gene expression seems to be associated with certain leukaemia types or subtypes. Several *HOXA* genes, namely *HOXA9* and *7* are overexpressed in a significant proportion of AML. *HOXC* genes, particularly *HOXC4* and *6*, are overexpressed in both ALL and AML. *HLX* and *DLX* homeobox genes were also found up-regulated in many cases of ALL and AML. On the other hand, a number of *HOX* genes were found down-regulated in APL (M3 AML), suggesting the link of HOX expression to PML-RARA. protein function. Members of *TALE* family (*MEIS1* and *PBX1*) were shown to collaborate with *HOX* genes in the induction of acute leukaemia and may accelerate the onset of leukaemic transformation mediated by *HOX* genes [Stamatoyannopoulos *et al* 2001, van Oostveen *et al* 1999, Buske *et al* 2000, Owens *et al* 2002, Thorsteinsdottir *et al* 2001, Drabkin *et al* 2002, Thompson *et al* 2003, Haga *et al* 2000, Kawagoe *et al* 1999 and 2001, Afonja *et al* 2000, Lawrence *et al* 1999].

A correlation between homeobox gene overexpression and clinical outcome has been observed, suggesting its prognostic value in acute leukaemia but further clinical studies are needed to confirm these findings [Drabkin *et al* 2002].



MSSLYY-N - aminoterminal region
C -connective hinge region
H - helix

Figure 2.11. NUP98–homeobox gene translocations.

2.1.2.11. FLT-3 and c-KIT alterations

FLT3

FLT3 (Fms-like tyrosine kinase 3) gene on chromosome 13q12 encodes the FLT3 receptor, a member of the class III receptor tyrosine kinase. It is preferentially expressed in haematopoietic stem cells and early progenitor cells and plays a central role in the growth regulation of early haematopoiesis. FLT3 consists of an extracellular region composed of five immunoglobulin domains, a transmembrane region, and an intracellular region that includes a juxtamembrane domain and two tyrosine kinase domains separated by an insert domain [Gilliland *et al* 2002a, Stirewalt *et al* 2003].

FLT3 alterations are the most frequent mutations in AML and relatively frequent (up to 30 %) in infant leukaemia and childhood ALL with hyperdiploidy.

They are of two main types, length mutations and point mutations. Different *FLT3* mutations may be present together, either simultaneously or consequently [Gilliland *et al* 2002a, Stirewalt *et al* 2003, Greaves *et al* 2006].

The most common aberration of *FLT3* gene is the internal tandem duplication (ITD). This mutation occurs in 5 – 20 % of childhood AML, with incidence increasing with the age, in about 20 – 25 % of adult AML, and around 15 % of secondary AML cases. It has been found in all AML subtypes, with the highest incidence in M3. Rarely, ITD occurs in ALL. The ITD mutations are clustered within exon 11 and 12 of *FLT3* gene, encoding the juxtamembrane domain. The duplicated fragments contain an in-frame directly oriented sequence of variable length (6 – 180 bp) corresponding to the region between amino acids 575 and 613 of the FLT3 protein (Figure 2.12.). Other length mutations have been described in rare cases, for example smaller insertions in the activation loop of the second tyrosine kinase domain.

The other, less frequent, alterations of *FLT3* gene are point mutations. Several different substitutions, all in the codon 835, have been described in approximately 7 % of AML and 3 % of ALL patients. Recently, another mutations in the codon 836 were identified: a deletion, and a substitution combined with insertion. All these point mutations are located in the activation loop of the second tyrosine kinase domain [Gilliland *et al* 2002a, Stirewalt *et al* 2003, Zwaan *et al* 2003].

Both length and point mutations cause gain-of-function alteration, a constitutive activation of the tyrosine kinase function. In case of point mutations this is supposed to be a direct result of the missense mutations within the functional tyrosine kinase domain. The mechanism of ITD effect is not quite clear. It has been shown that the length-mutated juxtamembrane domain plays an important role in the ligand-independent receptor activation. The juxtamembrane domain is supposed to induce the receptor interactions, homodimerization of FLT3 ITD mutated receptors and also heterodimerization of FLT3 ITD mutated receptor with wild type FLT3, in both cases leading to the receptor auto- and transphosphorylation and thus permanent activation. The tyrosine kinase then activates downstream signalling molecules, including RAS/MAPK, STAT5 and PI3K pathways, which are responsible for the transforming potential of *FLT3* mutations [Gilliland *et al* 2002a and b, Stirewalt *et al* 2001 and 2003, Kelly *et al* 2002, Reilly *et al* 2003, Kiyoi *et al* 2002, Spiekermann *et al* 2002, Yamamoto *et al* 2001, Meshinchi *et al* 2003, Anastasiadou *et al* 2003, Zwaan *et al* 2003].

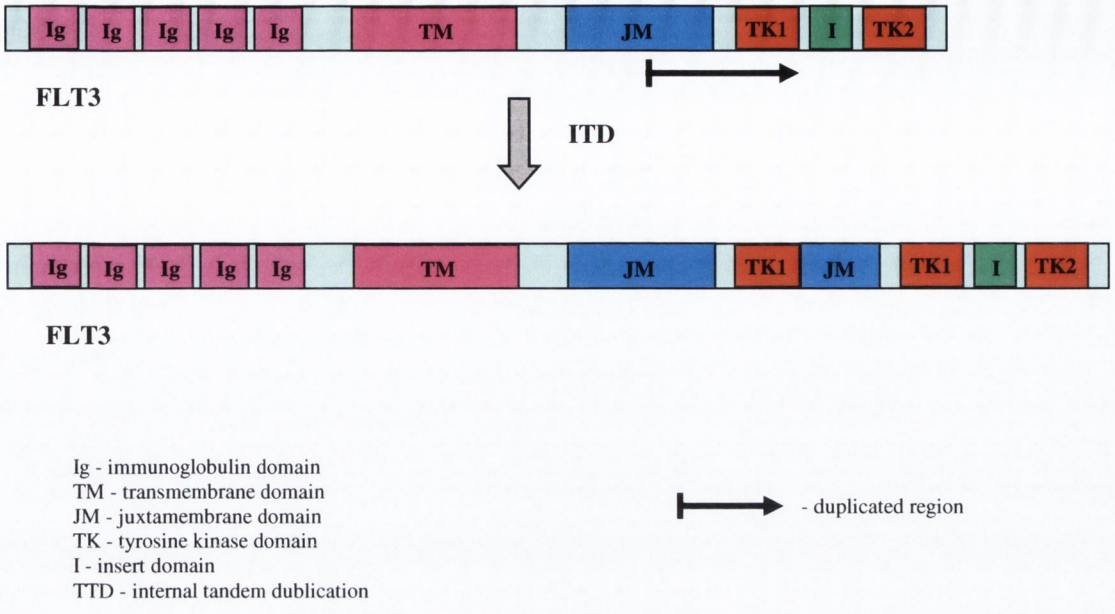


Figure 2.12. FLT3 internal tandem duplication.

The *FLT3* mutations are often detected in AML cases without other genetic alterations, although they may occur concurrently with many other gene rearrangements and point mutations. The exception is APL (M3 AML) with t(15;17), where the co-incidence with *FLT3* mutations is relatively high. In AML, *FLT3* ITD has been shown to be a strong independent prognostic factor associated with unfavourable outcome, especially in patients with no other chromosomal alterations. In some studies, the relative ratio between the mutated and wild *FLT3* expression rather than the presence of the mutation itself has been shown to act as the independent negative prognostic factor. About one third of patients with *FLT3* ITD may show a loss of the other wild-type allele, eventually a mutation of the other allele. These patients have poorer prognosis than patient with heterozygous mutation and one intact *FLT3* allele. The prognostic relevance of the point mutations is not quite defined but seems to have similar unfavourable impact. *FLT3* mutations were also correlated with a higher leukocyte count [Thiede *et al* 2002, Schnittger *et al* 2002, Kainz *et al* 2002, Liang *et al* 2002 and 2003, Arrigoni *et al* 2003, Moreno *et al* 2003, Noguera *et al* 2002, Boissel *et al* 2002, Christiansen *et al* 2001, Kottaridis *et al* 2001, 2002 and 2003, Meshinchi *et al* 2001, Kondo *et al* 1999, Xu *et al* 2000, Iwai *et al* 1999].

As *FLT3* mutations are the most frequent mutations in AML, they have been considered as a new potential marker for minimal residual disease monitoring. Unfortunately, it seems that they represent rather secondary events in leukaemogenesis with unstable expression pattern during the disease course [Schnittger *et al* 2002, Kottaridis *et al* 2002, Shih *et al* 2002].

In ALL, *FLT3* alterations were found to be associated particularly with MLL gene rearrangement, including MLL internal tandem duplication. They comprise either overexpression of wild-type FLT3 or previously described activating mutations. Their prognostic impact is not clear [Armstrong *et al* 2003].

FLT3 ITD may be detected from DNA or RNA, each having some advantages and disadvantages [van der Velden *et al* 2003, Murphy *et al* 2003, Stirewalt *et al* 2001, Kottaridis *et al* 2002]. Apart from bone marrow or peripheral blood cells, the detection of Flt3 ITD is possible also from peripheral blood plasma DNA [Jilani *et al* 2003].

c-KIT

c-KIT on chromosome 4p12 is another member of the class III receptor protein tyrosine kinases, in which genetic alterations have been associated with malignant myeloid disorders. *c-KIT* is expressed in early myeloid progenitor cells and mediates signals important for proliferation, survival, differentiation and adhesion. The structure of the receptor is similar to that of FLT3; it consists of an extracellular region composed of five immunoglobulin domains, a transmembrane region, and an intracellular region that includes a juxtamembrane domain and two separated tyrosine kinase domains [Kelly *et al* 2002, Meshinchi *et al* 2003].

Mutations causing constitutive activation of *c-KIT*, similar to *FLT3*, were identified in about 5 % of AML and are found also in other haematologic malignancies and solid tumours. These mutations can be classified into two types. Mutations in the enzymatic pocket affect the activation loop at the entrance to the enzymatic pocket formed by the split intracellular kinase domain. These substitution point mutations may affect several sites, in the case of AML mostly the codon 816, and may not be accessible with inhibitors competing with wild-type enzymatic sites. Mutations outside this region affect portions of the molecule that regulate the kinase activity. Such mutations can be in the juxtamembrane domain and cause release of a normally inhibitory domain, resulting in constitutive tyrosine kinase activation. In AML, these are again mostly substitution point mutations (insertions are found in gastrointestinal stromal tumours); small deletions may rarely occur. In contrast to the first type of mutations, regulatory mutations are accessible to ATP competition type of tyrosine kinase inhibitory therapy (like imatinib). Constitutively active c-KIT then stimulates downstream molecules like PI3K-AKT or STAT3 pathways. Prognosis of these mutations has yet to be established [Arrigoni *et al* 2003, Kelly *et al* 2002, Meshinchi *et al* 2003, Anastasiadou *et al* 2003, Clark *et al* 2003].

Deletions and point mutations were also found in another receptor tyrosine kinase, c-FMS, particularly in M4 subtype [Clark *et al* 2003].

Activating mutations in the transmembrane domain have been also detected in non-tyrosine kinase receptors, such as G-CSF receptor, in AML [Forbes *et al* 2002]. This suggests the general and important role of mutations affecting receptors involved in proliferation and differentiation signalling in the development of acute leukaemia.

2.1.2.12. Tumour suppressor genes

p15, p16, p18 and p19

All these tumour suppressor genes are cyclin-dependent protein kinases (Cdk) inhibitors and are altered at variable frequency in haematological malignancies. *p16* and *p15*, both on chromosome 9p21, are relatively often affected. The most frequent alterations are homozygous deletions of *p16* (*MTS1*),

usually with co-deletions of *p15* (*MTS2*) that can be identified in up to 30 % of ALL, both precursor B and T (20 % of B-ALL and 80 % of T-ALL). The prevalent mechanism responsible for 9p21 deletions in ALL is probably illegitimate V(D)J recombination. A preferential loss of the maternal 9p allele has been found, suggesting the possible role of germ-line events on the onset of childhood ALL. Inactivation by hypermethylation of the *p15* and *p16* promoters also occurs, while point mutations of *p16* or *p15* in leukaemia are, in contrast to solid tumours, quite rare. In AML, inactivations of *p16* or *p15* are uncommon. Inactivation of *p15* and *p16* has been shown to be associated with higher risks and unfavourable prognosis. A single deletion of 9p is regarded as an adverse prognostic factor in B-ALL, homozygous deletions are significantly associated with poorer prognosis in all types of leukaemia [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Shah *et al* 2001, Tutor *et al* 2002, Dalle *et al* 2002, Wong *et al* 2000, Harrison *et al* 2002, Guo *et al* 2000, Kitagawa *et al et al* 2002, Einsiedel *et al* 2001, Krug *et al* 2002, Zuna *et al* 2002, Morison *et al* 2002, Tschan *et al* 2001, Graf *et al* 2001, Carter *et al* 2001].

Deletions or hypermethylations of *p18* and *p19* occur in acute leukaemias infrequently [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Krug *et al* 2002].

p53 and p73

p53 is the most frequently mutated tumour suppressor gene in human malignancies, but is more prevalent in solid tumours than in leukaemias. Inactivated *p53* can be found in about 10 - 15 % of AML, 2 % of B-ALL (with higher frequency in L3 B-ALL and Burkitt's lymphoma) and 5 % of T-ALL.

p53 gene is located on chromosome 17p and encodes a 393-amino acid sequence-specific transcription factor that plays a critical role in activating expression of genes involved in cell cycle arrest and apoptosis as a response to DNA damage. The mechanism of inactivation of *p53* in haematopoietic neoplasms is similar to that in solid tumours and occurs predominantly through point mutation in one allele and deletion of the other allele. In addition to this classic mechanism, mutant p53 can lead to formation of dominant-negative p53 mutants that transform by acting as inhibitory proteins interfering with the normal functions of the wild-type p53 protein. Similar to solid tumour, *p53* mutations in leukaemias are mostly clustered from exon 5 to 10, which corresponds to the DNA-binding domain, and are mostly missense mutations. The mutant forms of p53 are accumulated in the cells and may be detected also by monoclonal antibodies anti-p53. In leukaemia, inactivation of *p53* appears to be linked with advanced stage of the disease and poor prognosis. It is very rarely found in ALL and AML at diagnosis, but is more frequent in relapses [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Krug *et al* 2002].

p73 is a new putative tumour suppressor gene related to *p53*. It is inactivated by hypermethylation in approximately 30 % of B-ALL, suggesting a role for *p73* in the development of B-ALL (mainly pre-B-ALL) [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Krug *et al* 2002].

ATM

ATM (ataxia-telangiectasia mutated) gene, located on chromosome 11q22-23, encodes a member of a family of protein kinases acting as a key regulator of multiple signalling cascades, including p53 pathway, that respond to DNA strand breaks induced by damaging agents or by normal processes, such as meiotic

or V(D)J recombination. Deletion and point mutation are the most common mechanisms of *ATM* alteration. This gene is altered in some cases of ALL. Its consistent inactivation occurs in patients with ataxia telangiectasia (AT), who have strong predisposition to development of lymphoid malignancies, including ALL. Patients with AT frequently possess chromosomal translocations with breaks in one of the *TCR* loci. These translocations may be present in large clones of T cells and may persist for many years without any clinical manifestation. However, a subset of patients eventually develops T-ALL, often T-cell prolymphocytic leukaemia, which is the most common malignancy occurring in ataxia telangiectasia syndrome. Three particular rearrangements are associated with T-ALL in AT patients: inv(14) and t(14;14), which deregulate *TCL1* expression, and t(X;14), which deregulates *MTCP1* expression [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001].

RB1

RB1 gene on chromosome 13q14 is one of the first tumour suppressor genes identified. The retinoblastoma gene encodes a nuclear phosphoprotein that acts as a cell cycle control check-point in the G1 phase. *RB1* may be inactivated by point mutations, deletions or binding to transforming viral proteins encoded by DNA viruses like adenovirus and human papilloma virus. Inactivation of *RB1* can lead to deregulation of cell cycle control and malignant transformation. In acute leukaemia, alterations of *RB1* are rather rare and show an association with unfavourable prognosis [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Markaki *et al* 2001, Guo *et al* 2000].

2.1.2.13. RAS genes

RAS proteins are key components of many signalling pathways that regulate cell proliferation, differentiation and apoptosis. Mutations of *RAS* family of protooncogenes can be found in many haematological malignancies, including ALL and AML, as well as solid tumours. *K-RAS* and *N-RAS* are deregulated in about 10 – 30 % of acute leukaemias of both types.

K-RAS and *N-RAS* genes on chromosome 12p are altered by point mutations of specific codons (12, 13 and 61) resulting in amino acid substitutions. This change leads to a constitutive activation of RAS that remains in the GTP-bound active state regardless of regulatory signals. The consequent result is a deregulated activation of multiple signal transduction pathways, leading to abnormal activation of transcription factors and gene deregulation.

RAS pathway is also affected in neurofibromatosis type 1, a disease with a hereditary inactivating mutation of *NF-1*, which is GTPase activating protein (GAP). Loss of GAP function is responsible for a prolonged or permanent RAS activation. This condition is associated particularly with juvenile myelomonocytic leukaemia [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, Rebollo *et al* 1999, Meshinchi *et al* 2003, Kiyoi *et al* 1999, Bowen *et al* 2003].

2.1.2.14. NOTCH genes

The *NOTCH* receptor family consists of four members, *NOTCH1-4* located on chromosomes 9q34, 1p11-13, 19p13 and 6p21, respectively, that play an important role in cell fate determination during development. These genes encode transmembrane receptors which are formed by an extracellular domain consisting of many EGL(epidermal growth factor)-like and several Lin12/Notch repeats; the transmembrane domain; and the intracellular domain (ICN) consisting of a RAM domain, ankyrin (ANK) repeats, nuclear localization sequences (NLS, missing in NOTCH4), and proline-glutamate-serine-threonine-rich (PEST) domain. The ICN and transmembrane domain of NOTCH1 are also called TAN-1. The RAM domain is the primary binding site for transcriptional regulator CBF1/RBPJκ. ANK repeat domain can also interact with this protein and, apart from that, binds other regulatory proteins like Deltex, Mastermind and Numb, which modulate NOTCH signalling. After the receptor activation, the ICN is released by proteolytic cleavage of gamma-secretase and act as a transcriptional co-regulator. It binds to the transcriptional repressor CBF1/RBPJκ and transactivates CBF1-regulated genes such as HES1 (hairy and enhancer of split). The activity of ICN may be modulated by interactions with additional regulators, including the positive regulators Deltex and Mastermind, or negative regulator Numb. The precise mechanism of NOTCH action and interaction with CBF1 is not quite understood. It is believed that CBF1 functions as a transcriptional repressor when interacting with the co-repressor/histone deacetylase complex (HDAC), and it changes into an activator by binding the co-activator ICN, or by other, not known mechanisms. Its main target gene, HES1, encodes a transcription factor of basic helix-loop-helix family that regulates, in general suppresses, expression of other genes [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Kojika *et al* 2001, Kawamata *et al* 2002, Izon *et al* 2002, Singh *et al* 2000, Milner *et al* 1999, Carlesso *et al* 1999, Allman *et al* 2002, Aster *et al* 2005].

NOTCH1 signalling is essential for normal T-cell development. Mutations of this gene (short deletions or duplications, point mutations) are commonly (in about 50 %) detected in T-ALL and are of 2 main types: extracellular domain mutations that increase ICN production, and intracellular C-terminal mutations that sustain ICN action. The gene is also affected in t(7;9)(q34;q34) translocation, that rearranges *NOTCH1* on chromosome 7 to *TCRB* locus on chromosome 9. This translocation is rarely (in about 1 %) detected in T-ALL. The breakpoints occur within 100 bp of an intron in *NOTCH1*, resulting in the formation of a truncated transcript and truncated protein consisting only of the intracellular domain. This truncated NOTCH1 protein has been shown to have transforming properties. It is located in the nucleus instead of the cell surface and is probably constitutively active, binds CBF1 and dysregulate transcription of CBF1 target genes. Inhibitors of ICN production and activity abrogate the growth of established T-ALL cell lines and are getting into clinical trials for patients with refractory T-ALL [Knowles *et al* 2001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001, Aster *et al* 2005, Grabher *et al* 2006].

2.1.2.15. IL-3

Interleukin 3 is one of the most important haematopoietic growth factors regulating the development of all lineages. The gene coding for IL-3 is affected in rarer cases of B-ALL, of pre-B subtype. The translocation t(5;14)(q31;q32) rearranges *IL3* gene on chromosome 5 to immunoglobulin heavy chain (IgH) gene on chromosome 14. This results in overexpression of IL-3 due to dysregulation of transcription, which is mediated by the active enhancer and promoter of the *IGH* gene. The excessive production of IL-3 forms a potent autocrine loop stimulating the cells affected with the aberration themselves and inducing their abnormal proliferation [Knowles *et al* 001, Degos *et al* 2000, Stamatoyannopoulos *et al* 2001].

2.1.2.16. JAK-STAT

JAK-STAT signalling is one of the crucial pathways in haematopoietic cells and alterations affecting these molecules may result in serious deregulation of cellular processes, proliferation in particular. JAK, a receptor-associated tyrosine kinase, is affected in the translocation t(9;12)(p24;p13), rarely found in ALL. Here the *JAK2* gene on chromosome 9 is rearranged to *TEL* gene on chromosome 12. The result of this translocation is a fusion protein with a dysregulated permanent JAK2 tyrosine kinase activity [Stamatoyannopoulos *et al* 2001, Harrison *et al* 2002, Lin *et al* 2000].

2.1.2.17. GATA-1

GATA-1, a member of GATA family of transcription factors, is necessary for normal haematopoiesis. It is required for normal erythroid and megakaryocytic development and differentiation. Inherited missense mutations in *GATA-1* have been found in inherited blood disorders (X-linked dyserythropoietic anemia, X-linked thrombocytopenia and beta-thalassemia) while acquired mutations are associated with essentially all cases of acute megakaryoblastic leukaemia in children with Down syndrome and the closely related transient myeloproliferative disorder. The latter mutations lead to the expression of a shorter isoform of GATA-1 named GATA-1s, which retains both DNA binding zinc fingers but is missing the N-terminal transactivation domain. It has been predicted that the inability of GATA-1s to regulate its corresponding target genes in megakaryocytes represents the main mechanism responsible for its leukaemogenic potential and also the special characteristics of this type of leukaemia (e.g. its remarkable hypersensitivity to cytosine arabinoside due to insufficient expression of cytidine deaminase) [Cantor *et al* 2005, Crispino *et al* 2005, Muntean *et al* 2006, Harigae *et al* 2006].

2.1.2.18. Regulators of apoptosis

The alterations affecting BCL-2 family members are not as common in acute leukaemia as in other haematological malignancies (chronic lymphoproliferations) but may contribute in a significant way to the leukaemic process. The up-regulation of the anti-apoptotic members, like BCL-2, or down-regulation

of the pro-apoptotic members, like BCL-x_S, have been correlated to unfavourable outcomes in acute leukaemia (AML) and have been proposed as potential prognostic factors. The principal proposed mechanism of their action is by mediating resistance to chemotherapy-induced apoptosis. Mutations of these genes in association with acute leukaemia have not been described [Karakas *et al* 2002, Yamaguchi *et al* 2002].

2.1.2.19. Other genes and proteins

Telomerase

Telomerase function is necessary for the permanent proliferation and immortality of malignant cells. An increased telomerase expression and activity has been observed in childhood ALL and AML and seems to be correlated with worse response to the therapy, though this has not been proven in a larger clinical study to date [Malaska *et al* 2000].

ABC proteins

The ABC (ATP-binding cassette) superfamily of transport proteins involves several groups of proteins that are responsible for cellular drug resistance. The most extensively studied members are P-170 and related proteins MRP and LRP. More recently, another important member, BCRP, has been identified.

Multidrug resistance gene (*MDR1*) on chromosome 7 encodes a phosphoprotein, P-glycoprotein 170 (P-170). This membrane protein, that functions as an active pump, is responsible for accelerated efflux of various cytostatic drugs out of the malignant cells and of apoptotic resistance of the cells. Other, drug-efflux independent mechanisms may also be involved. MDR1 significantly contributes to the resistance of leukaemic cells to the cytostatic therapy and the treatment failure. Other similar proteins that may play a role in this multidrug resistance mechanism and be potentially implicated in leukaemia drug resistance are multidrug resistance-associated protein 1 (MRP1) and lung-resistance protein (LRP). The expression of MDR1 and the related proteins has been found in up to 10 – 20 % of acute leukaemias, both ALL and AML. In several studies involving childhood ALL and adult AML, the expression of these genes was associated with resistance and an unfavourable prognosis and *MDR1* is suggested to be another independent adverse prognostic factor. The prognostic value in childhood AML has not been found [Tafuri *et al* 2002, Steinbach *et al* 2003, Pallis *et al* 2003].

The *ABCG2* gene on chromosome 4, is a member of the ABCG subfamily, encodes the breast cancer resistance protein (BCRP), also known as mitoxantron resistance protein (MXR) or placenta ABC protein (ABC-P). BCRP expression has been detected in childhood ALL (in low frequency) and AML (higher frequency). In AML, including childhood AML, the BCRP expression was found to correlate with worse treatment response and higher relapse rate, while in ALL no significant correlation with the prognosis or disease response has been observed [Sauerbrey *et al* 2002, Steinbach *et al* 2002a].

Ikaros gene

Ikaros gene, a member of a family of highly conserved homologous proteins, encodes at least six alternatively-spliced isoforms (IK1-6) of a zinc finger transcription factor with an important role in

regulation of lymphoid proliferation and differentiation. The protein consists of two functionally distinct zinc finger domains: N-terminal sequence-specific DNA binding domain and C-terminal oligomerization domain. The number of zinc fingers in the N-terminal domain confers different DNA binding activities of different isoforms. Ikaros proteins may act both as a transcriptional repressors and activators through their ability to bind a large number of nuclear factors, including components of histone deacetylase and ATP-dependent chromatin remodelling complexes. Their function is determined by the interactions with other molecules, among them the most important are other family members (Aiolos, Helios, Eos, Pegasus), histone deacetylase repressor complexes NURD and SIN3, and the nucleosome remodelling complex SWI/SNF. Because of the nature of these binding partners, much of the influence of Ikaros upon transcription is thought to be mediated through chromatin reorganisation. Ikaros proteins are localised to discrete nuclear foci and this localisation is important for their function [Westman *et al* 2002, Georgopoulos *et al* 2002, Schmitt *et al* 2002].

Aberrant Ikaros expression has been linked with infant ALL and T-ALL. In these diseases, either overexpression of dominant negative isoforms or a reduced level of Ikaros activity, as well as various insertions and deletions adjacent to the C-terminal domain of the gene, has been observed. Also, different isoform expression patterns have been detected in different leukaemia subsets. The molecular mechanisms of Ikaros function need further elucidation [Westman *et al* 2002, Georgopoulos *et al* 2002, Schmitt *et al* 2002, Olivero *et al* 2000].

PRAME

PRAME (preferentially expressed antigen of melanoma) gene encodes a protein with unknown function that is recognised as a tumour antigen by cytotoxic T cells. This protein, expressed in various tumours, was detected in a proportion of ALL and AML cases, but also in normal haematopoietic stem cells. In some studies, higher levels of *PRAME* expression correlated with favourable prognosis and increased levels of *PRAME* expression could be found to precede clinical relapse thus suggesting its possible use in minimal residual disease monitoring. However, there is currently not enough data to confirm these observations [Matsushita *et al* 2001, Steinbach *et al* 2002b].

CREB

Cyclic adenosine monophosphate (cAMP) response-element binding protein (CREB), a product of the gene located on chromosome 2q32-34, is a nuclear protein that regulates gene expression on activation of cAMP-dependent or cAMP-independent signal transduction pathways. Its function is modulated by serine phosphorylation in response to growth factor stimulation. CREB binds to the cAMP response element regulatory sequences of target genes regulating cell proliferation and survival, such as Bcl-2 and Egr-1, and is one of critical regulators of growth factor-induced gene expression leading to cell proliferation, differentiation and survival. Overexpression of CREB was described in high frequencies in patients with both ALL and AML while it was not found in normal bone marrow samples. The overexpression may be caused by translocations affecting 2q32-34 but in most cases other, not well understood mechanisms, are involved. The role of *CREB* in leukaemogenesis is to be defined. Antisense oligonucleotides to *CREB*

were shown to induce apoptosis of human leukaemia cells *in vitro* [Crans-Vargas *et al* 2002, Biondi *et al* 2000a].

CBP/p300

CBP (CREB-binding protein) and p300 are transcriptional adapters and co-activators of transcription factors and thus important regulators of transcription. CBP, encoded by a gene on chromosome 16p13, is affected in translocations like t(11;16)(q23;p13) with *CBP-MLL* fusion or t(8;16)(p11;p13) with *CBP-MOZ* fusion; *p300* on chromosome 22q13 is rearranged in similar translocations t(11;22)(q23;q13) to *MLL*, and t(8;22)(p11;q13) to *MOZ*, and other translocations involving *CBF* or *p300* have been described [Biondi *et al* 2000a, Dash *et al* 2001, Blobel *et al* 2000].

C/EBP

CCAAT/enhancer binding protein α (C/EBPα) is a crucial transcription factor for differentiation of mature granulocytes. Recently, different *C/EBPα* mutations likely to induce differentiation arrest have been described in about 7 - 10 % of AML, more often associated with M1 or 2 subtypes. C-terminal mutations are usually in-frame and affect the basic leucine zipper domain, resulting in deficient DNA binding. In most cases they are associated with frameshift mutations in the N-terminus. Presence of these mutations was identified as a potential independent good prognostic factor but further studies are required to confirm this observation [Preudhomme *et al* 2002, van Waalwijk *et al* 2003].

VEGF

Angiogenesis is a crucial event in the carcinogenesis and is necessary for the growth and metastatic spread of solid tumours. Recently, it has been also demonstrated its importance in the development and progression of leukaemia including acute leukaemia. Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors. It signals via receptor tyrosine kinases, including VEGF-R2 receptor, which may be present on leukaemic blasts. Increased bone marrow angiogenesis and VEGF levels were shown to be adverse prognostic features in patients with AML [Giles *et al* 2003].

Other potential genes

Human neuronatin (*NNAT*) gene, mapped to 20q11-12, is an imprinted gene normally expressed in brain but also in haematopoietic progenitors. Substantial or complete loss of the unmethylated *NNAT* allele combined with hypermethylation of the other one were found to be a frequent event in both ALL and AML. The importance of this epigenetic mechanism of dysregulation of genes at 20q11-12 in leukaemia development needs further studies [Kuerbitz *et al* 2002, Clark *et al* 2003].

The loss of expression of the deleted in colorectal carcinoma (*DCC*) gene has been found in proportion in AML patients and shown to be an adverse prognostic factor [Inokuchi *et al* 2002].

2.1.3. Clonal rearrangements of antigen receptor genes in childhood acute leukaemia

2.1.3.1. Immunoglobulin and T-cell receptor gene rearrangements in normal lymphoid cells and leukaemia

The specific antigen recognition mediated by lymphocytes depends on the presence of specific effector molecules on the surface of the cells, immunoglobulin receptors (IGs, also called B-cell receptors, BCRs) and T-cell receptors (TCRs). They are members of the *IG* gene superfamily with unique and remarkably complex structure and functional evolution. The repertoire of IGs and TCRs must allow the recognition of an extraordinarily large number of discrete antigen epitopes and must be appropriately selected in terms of self-recognition and self-nonself discrimination. This extensive and specific repertoire of IGs and TCRs is generated during B- and T-cell differentiation by a complex process of multiple gene rearrangements. In the antigen-independent period of differentiation, the basic IGs or TCRs repertoire is generated by primary *IG/TCR* gene rearrangements. During the following antigen-dependent period, a second level of diversity is generated as a result of secondary rearrangements and, in case of *IGs*, somatic mutations. *IGs* then undergo isotype switching as the final differentiation step [Knowles *et al* 2001, Degos *et al* 2000].

As leukaemia is a disease with a clonal character, the vast majority of ALL (> 98 %) and even a small proportion of AML (10 %) cases contain identically (clonally) rearranged *IG* and/or *TCR* genes [van Dongen *et al* 2003, Boeckx *et al* 2002].

2.1.3.2. Process of IG/TCR gene rearrangements

The structure of the *IG* and *TCR* genes is similar. The *IGH* gene and *TCRB* and *TCRD* genes consist of clusters of variable (V), diversity (D), joining (J) and constant (C) regions; *IGL* (*IGK* and *IGL*) genes and *TCRA* and *TCRG* genes consist of clusters of V, J and C regions. The regions may contain one to over hundred segments, some of them being functional, the rest non-functional (pseudogenes), that are categorised into families. Members of the particular family show high sequence homology and contain a consensus conserved nucleotide sequence [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 2003].

The process of rearrangement of the antigen receptor genes during early lymphoid differentiation gives rise to the vast repertoire of specific IG and TCR molecules of B and T cells. The extent of the whole repertoire of the antigen receptors is determined by the diversity of the germ-line genome (the number of V, D, J and C segments), the combinatorial diversity (number of possible V(D)J combinations) and the junctional diversity (variability of the sequences joining the V, D and J segments). In B cells, further diversification and refinement are achieved by isotype switching recombination (recombination of C segments), somatic hypermutation and receptor editing (secondary V(D)J recombinations) (Figure 2.13.).

All loci encoding the IG and TCR chains undergo the somatic DNA primary V(D)J rearrangements to form a complete variable domain. This process of recombination, random association of the gene segments, occurs in a highly ordered fashion. In B cells, *IGH* genes rearrange first, then *IGK*. This may be

followed by *IGK* deletion and *IGL* rearrangement. In T cells, *TCRD* genes rearrange first, then *TCRG*. This may be followed by *TCRD* deletion with *TCRB* rearrangement and subsequently *TCRA* rearrangement. In case of *IGH* and *TCRB* and *TCRD*, the first event is an association of one D to one J gene segment followed by joining of one V segment to the recombined DJ segment. In case of *IGK* and *IGL* and *TCRA* and *TCRG*, the rearrangement consists of the recombination of a V segment to a J segment. *TCR* genes rearrange only in T cells, *IG* genes only in B cells (except for D-J_H which may be present also in T cells).

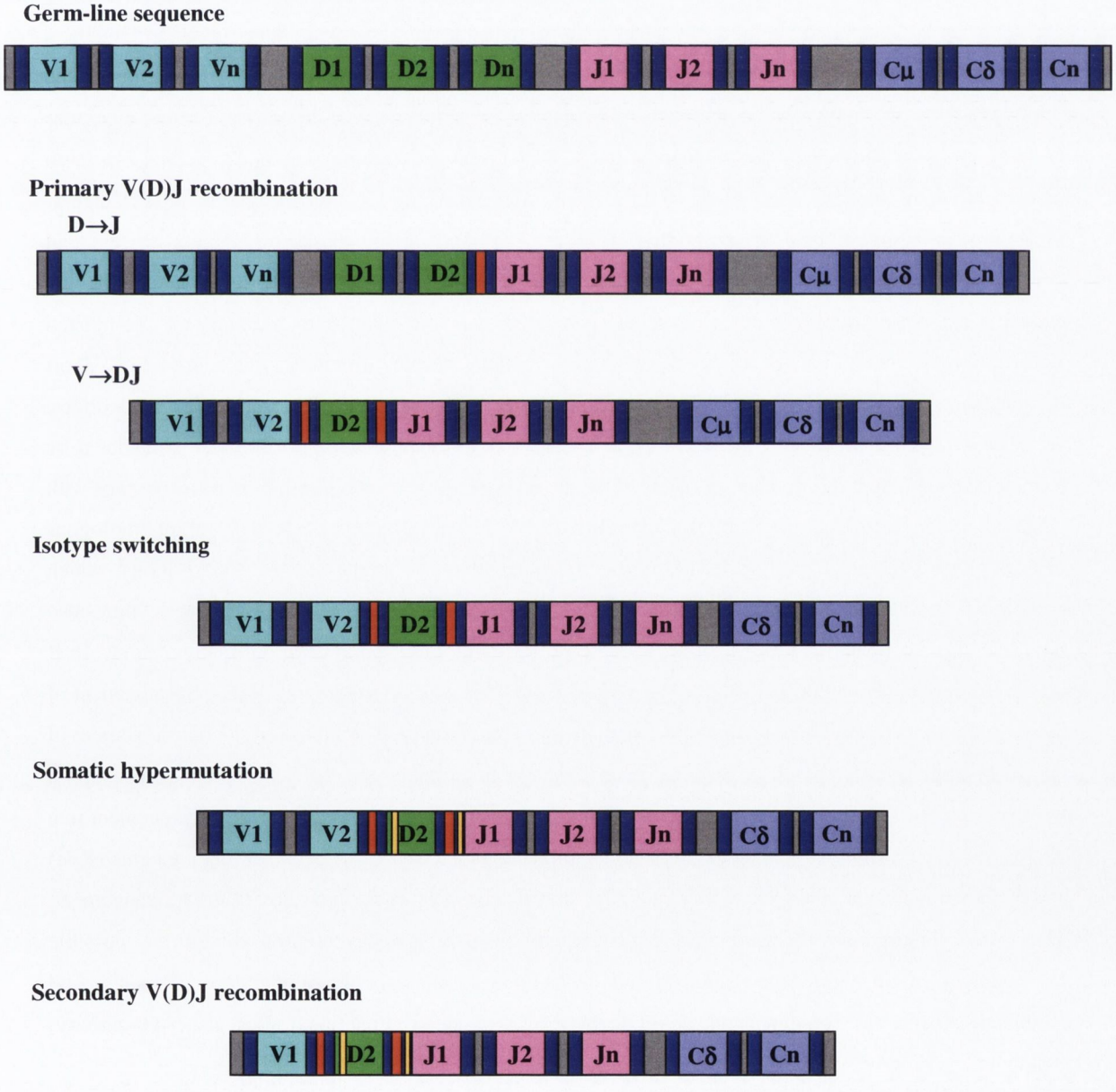


Figure 2.13. IG rearrangements.

The recombination is accurately targeted by characteristic recombination signal sequences (RSS) flanking each of the V, D and J gene segments (being localised downstream of the V gene segments, at both sides of the D gene segments and upstream of the J gene segments). RSS consist of conserved palindromic heptamer and AT-rich nonamer sequences separated by a non-conserved 12 or 23 nucleotide spacer. Inappropriate (mutated or crippled) RSS reduce or completely prevent the rearrangements. The V(D)J recombination is initiated by DNA cleavage by lymphoid-specific recombinases encoded by genes RAG-1 and 2 (recombination-activating genes). They recognise the RSS and, providing the target locus has an open or accessible conformation, catalyse the DNA cleavage precisely at the border between RSS and coding segment. The recombination is restricted to the proper pairs of sites (V to J, or V to D to J regions) between an RSS with a 12-bp pacer and one with a 23-bp space (the 12/23 rule). This feature prevents non-productive rearrangements (like V to V). After the cleavage step, the recombination is accomplished by reciprocal rejoining of the coding elements (coding joints) and the RSS heptamers (signal joints). Depending on the orientation of the RSS flanking the recombining gene segments, the rejoining of the two segments is associated with excision (the sequence is deleted in the form of a circular excision product, also called TCR excision circle (TREC) or B-cell receptor excision circle) or inversion of the intervening sequences in between them. Signal joints are recombined precisely, without a change of the germline sequence. In contrast, coding joints are modified during the recombination process and contain base losses and insertions. Bases losses result from random deletion of original nucleotides by exonuclease. Base additions are caused by templated and non-templated mechanisms. Templated insertions arise from off- centre opening of the sealed hairpin structure, which generates short complementary sequences (generally shorter than 4 bp) recognised as palindromic (P) nucleotides. Random template-independent insertion of new nucleotides (N nucleotides, up to 15 bp) is mediated by terminal deoxynucleotidyl transferase (TdT). The machinery required for rejoining the ends overlaps with general cellular pathways responsible for repairing double-strand DNA breaks (NHEJ – non-homologous end joining). These changes produce extreme sequence diversity at the site of V(D)J joints, which correspond to the antigen contact residues of IG and TCR molecules (CDRs). However, they also introduce the risk of non-functional genes because of out-frame joining and creation of stop codons. In B cells, *IGH* rearrangement occurs first, in the earlier stages of B cell differentiation. If the rearrangement of the first allele of *IGH* is non-functional, the second allele recombines. The failure of the rearrangement of the second allele leads to cell death. Conversely, once a functional gene has been obtained, it exerts a negative feedback on a further rearrangement. *IGH* rearrangement is followed by *IGK* rearrangement in a similar way. In case this is not successful, the kappa locus is excised by Kappa-deleting elements (Kde) and *IGL* rearrangement takes place. In T cells, the rearrangement of *TCRB* precedes *TCRA* rearrangement. The primary rearrangement gives rise to combinatorial diversity (represented by all the possible combinations of different gene segments and determined by the number of the gene segments) and junctional diversity (represented and determined by the number of junctional sites and TdT activity) [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 2003].

The V(D)J rearrangement in B cells is followed by isotype switching recombination. Before immune stimulation, the *IGH* locus of a B-cell contains a VDJ segment just upstream of the C_{μ}, and the cell expresses IgM and may simultaneously co-express IgD (due to mechanism of alternative RNA processing

and termination of transcription). After immune stimulation B-cells frequently undergo isotype class switching, a process where the C region of *IGH* is replaced by another one (C_γ, C_α or C_ε) resulting in different IGH isotype. It is a regulated non-homologous recombination; because the constant region clusters for other IGH isotypes are located 3' of C_μ and C_δ within the *IGH* locus, switching recombination results in deletion of DNA sequence. The recombination involves characteristic DNA regions termed switch regions (S), located at the 5' end of each constant region sequences (except C_δ). They interact with specific enzymes, different from those implied in VDJ rearrangements. S regions contain characteristic tandem arrays of short repetitive sequences that are G rich on the non-template strand but are not homologous to one another and there is no specific sequence identified in switch region breakpoints. Contrary to V(D)J recombination, switch recombination is thus region specific but not sequence specific, and does not require RAG-1 and RAG-2. The IG isotype switch does not affect antigenic specificity but permits a more diverse immune response by altering the effector function of the IG molecules.

B cells may further diversify their IG molecules by an antigen-induced somatic hypermutation in V regions and possibly other sites. The rate of mutations in germinal centre B cells increases to 1 in 1000 bp per division. Together with stringent antigen-driven selection this results in the production of antibodies with improved affinity and specificity for target antigens. Somatic hypermutations of *IG* genes are confined to a region within about 1.5 kb downstream of the promoter. The mutations are primarily point mutations, mostly substitutions (transitions more often than transversions), although nucleotide insertions or deletions may occasionally occur. Usually, only one DNA strand is modified. Certain sequences have been identified as potential hot spots. The question of somatic hypermutation in *TCRA* and *TCRB* genes remain controversial. The molecular mechanism of somatic hypermutation remains largely unknown. It seems that it is closely linked to transcription with possible important role of transcriptional enhancers and cis-acting DNA sequences. The role of DNA strand breaks and various DNA repair systems is investigated.

The rearranged V regions of functional *IGH* and *IGL* genes can be adapted or replaced by secondary V(D)J rearrangements. During these the DJ complexes may be replaced with complexes that are formed by joining an upstream D and downstream J gene segments. VJ complexes may be changed in the same way. V segments of VDJ complexes may be replaced with new upstream V segments. These recombinations are catalysed by the same enzymes as the primary rearrangements and may be productive (in-frame) or non-productive (out-of-frame). Secondary V(D)J rearrangements are supposed to be the crucial mechanism to prevent the emergence of autoreactive IG specificities. In immature B cells they are referred to as receptor editing, in mature B and T cells as receptor revision [Knowles *et al* 2001, Degos *et al* 2000, van Dongen *et al* 2003].

2.2. Clinical aspects of childhood acute leukaemia

Acute leukaemia is a malignant disease of haematopoietic system characterised by an uncontrolled clonal proliferation and accumulation of altered haematopoietic precursors at the early stages of their development in the bone marrow.

According to the character of the malignant cells, leukaemic blasts, acute leukaemia is divided into two main groups: acute lymphoblastic leukaemia (ALL) and acute non-lymphoblastic leukaemia (ANLL). The latter is represented mainly by acute myeloid leukaemia (AML) and includes also hybrid acute leukaemia and acute undifferentiated leukaemia.

Childhood leukaemia is leukaemia occurring in childhood, with the onset at the age up to 15 years. A special subgroup of childhood acute leukaemia is infant leukaemia that occurs in the first 12 months of life and has features distinct from the rest of the group.

Childhood acute leukaemia represents the absolute majority of childhood leukaemias. Chronic leukaemia in children is rare and is of myeloid type, chronic myeloid leukaemia (CML) [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001].

2.2.1. Acute lymphoblastic leukaemia in childhood

2.2.1.1. Demography

Acute lymphoblastic leukaemia is the most frequent paediatric malignancy comprising 25 – 35 % of all malignant diseases in childhood and over 80 % of childhood leukaemia cases. Its incidence (number of new cases per year) in European countries and North America is around 3 - 4 / 100 000 and in most of developed countries is increasing. The peak incidence is at the age between 2 to 5 years. Males are more frequently affected than females (except in infancy when females predominate) and white children are more often affected then black children (with more pronounced difference at younger ages) [Degos *et al* 2000, Knowles *et al* 2001, Harrison *et al* 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, www.nci.nih.gov].

2.2.1.2. Etiology and risk factors

In some cases of ALL, the malignant cells may be found in the blood at the time of birth suggesting a prenatal origin of the leukaemic clone. In twins, one can acquire leukaemia from the other prenatally due to shared placental blood circulation. In majority of cases, the malignant transformation occurs postnatally [Degos *et al* 2000, Greaves *et al* 2003].

Several conditions have been identified to be associated with a significantly increased incidence of ALL. These so called risk factors include inherited genetic and non-genetic conditions and external factors (Table 2.2.). The most serious among them is Down's syndrome, the trisomy of 21 chromosome, which is found in 2 % of childhood ALL. These risk factors, however, are present in a minority of childhood ALL cases; the vast majority of them occur without a detectable predisposing condition.

Recently, delayed exposure to common infections seems to be associated with increased risk factor of childhood ALL. It may represent the second, postnatal "hit" and though its mechanism is not quite clear, it seems that it may lead to selection of pre-leukaemic clones (an inflammatory response to the infection and released cytokines may transiently suppress normal haematopoiesis and/or induce apoptosis; in this situation the pre-leukaemic clone could have a distinctive survival and/or proliferative advantage; a regenerative expansion of the cells may result in secondary mutations either by chance or through genotoxic influence of oxidative stress accompanying the inflammatory response) [Degos *et al* 2000, Knowles *et al* 2001, Greaves *et al* 2006, www.nci.nih.gov].

Table 2.2. Risk factors of childhood ALL.

Risk factors of childhood ALL:	
genetic diseases	Down's syndrome
	Klinefelter's syndrome
	G-trisomy
	Schwachman's syndrome
	Bloom's syndrome
	neurofibromatosis
	ataxia telangiectasia
	affected twin sibling
inherited non-genetic conditions	advanced maternal age at birth
	previous history of maternal fetal loss
	perinatal asphyxia
	birth weight over 4.5 kg
external factors - physical	radiation – prenatal, postnatal
	electromagnetic field (?)
- chemical	previous chemotherapy
	environmental mutagenes
- biological	treatment with growth hormone
	growth hormone deficiency (?)
- infection	leukaemogenic viruses (?)
	aberrant immune response to a common viral infection (?)

2.2.1.3. Pathogenesis

ALL is a clonal disease, the malignant cells arise from a common precursor. The clonal precursor undergoes genetic changes (chromosomal and gene mutations) that cause inactivation of tumour suppressor genes and activation of proto-oncogenes. These changes lead to the consequent transformation

of the normal cells into the malignant leukaemic blasts. Although many genes with a critical role in the malignant transformation are well described, some steps of this process have not yet been fully understood.

2.2.1.4. Classification

ALL is classified according to its morphological features, immunophenotype, cytogenetic and molecular aberrations.

Morphological classification

Diagnosis of ALL is based on the presence of more than 25 - 30 % blasts in the bone marrow. The remission is achieved when the number of blasts in bone marrow falls down under 5 % (although it may still represent the total number of $10^{10} - 10^{11}$ persisting malignant cells).

According to FAB (French-American-British Cooperative Group) morphological classification, ALL is divided into three groups, L1 – L3, depending on the blast morphology (Table 2.3.). Many leukaemias however show certain morphological heterogeneity, which may sometimes cause problems with their classification [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, www.nci.nih.gov].

Cytochemistry has lost most of its importance in the diagnosis of ALL because of common use of more effective methods like immunophenotyping (Table 2.4.) [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, www.nci.nih.gov].

Table 2.3. Morphological classification of ALL.

FAB classification of ALL
L1 – small monomorphic type
L2 – large heterogenous type
L3 – Burkitt's cell type *)

*) ALL with FAB L3 morphology, c-Myc translocations and surface immunoglobulin (sIg) expression represent a systemic manifestation of Burkitt's or Burkitt's-like non-Hodgkin's lymphoma (NHL) and are treated as such; rare cases of ALL with FAB L3 morphology and c-Myc translocations but without sIg expression are considered to be and treated as B-ALL.

Immunological classification

Antigens expressed by the leukaemic cells allow distinction of the origin and the degree of maturation of the leukaemic clone. According to EGIL (European Group for Immunological Classification of Leukaemias) classification, ALL is divided in two broad groups, B-ALL and T-ALL, each having four subgroups (Table 2.5.). B-ALL forms the majority of ALL cases, 80 - 85 %, occurring equally in both genders. T-ALL comprises the rest of ALL cases, 15 - 20 %, and occurs predominantly in males.

A special subgroup of ALL (B- or T-lineage), called ALL with myeloid antigen expression (ALL-My +), may express 1 or 2 pan-myeloid markers without fulfilling the criteria for biphenotypic acute leukaemia [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, www.nci.nih.gov].

Table 2.4. Cytochemistry of ALL.

Cytochemistry of ALL	
myeloperoxidase	–
Sudan black B	–
esterase	–
PAS	+ (L1 – 2)
acid phosphatase	– / focally +

Table 2.5. Immunological classification of ALL.

EGIL classification of ALL	
Type	Criteria
B-lineage ALL	≥ 2 of the 3 markers present: CD19 + and/or CD79a + and/or CD22 most also TdT + (except B-I) and HLA-DR +
B-I – pro-B-ALL B-II – common ALL B-III – pre-B-ALL B-IV – mature B-ALL intermediate type – transitional pre-B-ALL	no other differentiation B-cell antigens expressed CD10 + c Igμ + c or s Igκ or λ + c and s Igμ +, s Igκ and λ –
T-lineage ALL	c or s CD3 + most also TdT +, HLA-DR –, CD34 –
T-I – pro-T-ALL T-II – pre-T-ALL T-III – cortical T-ALL T-IV – mature T-ALL group a: α/β + T-ALL group b: γ/δ + T-ALL	CD7 + CD2 + and/or CD5 + and/or CD8 + CD1a + s CD3 +, CD1a – TCR α/β + TCR γ/δ +

(c – cytoplasmic, s – surface)

Cytogenetic and molecular classification

Cytogenetic or molecular alterations are present in the majority of ALL cases. With the methods available at present it is possible to identify a clonal chromosomal or molecular abnormality in 75 – 90 % of childhood ALL cases and classify them according to the findings.

The chromosomal alterations detected are either numerical or structural. Often both are associated and present together.

Numerical alterations involve changes in chromosome number, its reduction by chromosomal loss or augmentation by chromosomal gain (Table 2.6.).

Structural alterations include translocations in the vast majority of cases, deletions, inversions and additions (Table 2.7.) [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Otten *et al* 2002, Berman *et al* 2000, Shusterman *et al* 2000, Harrison *et al* 2000, 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, www.nci.nih.gov].

Table 2.6. Numerical chromosomal alterations in childhood ALL.

Numerical chromosomal alterations in childhood ALL		
reduction:	pseudohaploidy	24 – 28 chromosomes
	severe hypodiploidy	32 – 37 chromosomes
	hypodiploidy	38 – 45 chromosomes
augmentation:	hyperploidy	47 – 50 chromosomes
		50 – 65 chromosomes
		> 65 chromosomes
	near-tetraploidy or tetraploidy	≤ 92 chromosomes

2.2.1.5. Prognostic factors

Prognostic factors are independent characteristics associated with a particular prognosis, the disease outcome (most important characteristics of which are the relapse rate and disease-free and overall survival). Some of these also act as predictive factors, which means they can predict the response to the certain type of therapy. In many cases, several prognostic factors are grouped and present together. Prognostic factors include different clinical and laboratory findings that reflect some of the biological features of the leukaemia and allow an estimation of the disease course, response to the treatment and risk of relapse. Their correlation with the disease outcome as independent prognostic variables has been tested by multivariate analysis. Prognostic factors are treatment dependent – improvements in therapy may diminish the importance of or abrogate some of them [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Otten *et al* 2002, Harrison *et al* 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, www. nci.nih.gov] (Table 2.8.).

Based on presence of these factors, patients with ALL are divided into groups with similar risk and prognosis, and receive corresponding treatment (see 2.2.1.8.).

Table 2.7. Structural chromosomal alterations in childhood ALL.

Structural chromosomal alterations in childhood ALL		
Chromosomal alteration	Gene(s) involved	Clinical characteristics
t(12;21)(p12-13;q22)	TEL–AML1	20 – 30 % of B-ALL good prognosis
t(1;19)(q23;p13)	E2A–PBX1	5 % of ALL, mostly B-ALL associated with a high WBC unfavourable prognosis
t(9;22)(q34;q11)	BCR–ABL (Ph chromosome)	5 % of ALL, mostly B-ALL associated with high risk factors unfavourable prognosis
t(4;11)(q21;q23)	MLL–AF4	5 % of ALL, mostly infant B-ALL associated with high risk factors dismal prognosis
t(11;19)(q23;p13) t(9;11)(p21;q23)	MLL	rare, B- and T-ALL unfavourable prognosis
t(8;14)(q24;q32-3) t(2;8)(q12;q24) t(8;22)(q24;q11)	MYC–IgH MYC–Igλ MYC–Igκ	B-ALL of L3 type, NHL unfavourable prognosis
dic(9;12)(p11-13;p11-12) dic(9;20)(p1;q11)		1 – 2 % of B-ALL, male predominance good prognosis
del(9)(p21)	p16	around 10 % of ALL, mostly T-ALL associated with high risk factors unfavourable prognosis
t(5;14)(q31;q32)	IL-3–IgH	rare, B-ALL unfavourable prognosis
t(1;14)(p32;q11) microdel (1)(p32) t(8;14)(q24;q11) t(10;14)(q24;q11) t(11;14)(p13;q11)	TAL1–TCRδ SIL-TAL1 MYC–TCRα or δ HOX11–TCRα or δ TTG2–TCRα or δ	around 10 % of T-ALL, mostly in males associated with a high WBC
t(7;9)(q34;q32)	TCRβ–TAL2	T-ALL, rare

(t - translocation, del - deletion, inv - inversion, der - derivative, dic - dicentric)

Table 2.8. Prognostic factors in childhood ALL.

High risk prognostic factors in ALL	
patient characteristics - age - race - gender	 infants < 1 y adolescents > 10 y black male
clinical presentation - leucocyte count - organ involvement	 WBC > 50 x 10^9/l at diagnosis lymphomatous presentation (hepatomegaly, splenomegaly, mediastinal mass or lymphadenopathy CNS leukaemia
biological characteristics - FAB morphologic group - immunophenotype - cytogenetic and molecular aberrations	 L3, granular ALL T-ALL, B-ALL CD10 – near-tetraploidy, hypodiploidy < 45 translocations t(9;22), t(4;11), t(8;14), t(2;16), t(11;19), t(1;19)
treatment response - PB blasts after 7 days of steroids or multiagent chemotherapy - BM blasts at D8, D15 and the end of induction - MRD at the end of induction - in vitro survival of blasts in culture	 PB blasts present at D7 BM blasts > 5 % on D15 and D28 MRD present on D28
Low risk prognostic factors in ALL	
biological characteristics - immunophenotype - cytogenetic and molecular aberrations	 pro-B-ALL, CD10 +, CD34+ and CD45 – trisomies 21, 4, 10, 17 hyperploidy > 50 chromosomes DNA index > 1.16 translocation t(12;21)
treatment response	PB blasts absent at D7 BM blasts < 5 % on D15 and D28 MRD absent on D28

D – day, y – year(s), PB – peripheral blood, BM – bone marrow, MRD – minimal residual disease

2.2.1.6. Manifestation and course of the disease

The clinical presentation of ALL is a result of bone marrow infiltration suppressing normal haematopoiesis, eventually extramedullary spread with organ infiltration.

Bone marrow infiltration manifests with symptoms of anaemia (fatigue, pallor, dyspnoea), neutropenia (fever, stomatitis, infections) and thrombocytopenia (spontaneous bruises, purpura, bleeding).

Extramedullary spread involves predominantly lymphatic organs and manifests as lymphadenopathy, splenomegaly, hepatomegaly and skeletal pain. CNS involvement causing headache and neurological symptoms and testicular infiltration causing swelling and tenderness of the testicle are less frequent. Rarely, the leukaemic infiltrates may affect the ovary or, in infants, the skin and eyes.

The course of an untreated disease is usually characterised by a rapid progress. Infection and bleeding are the most common causes of death [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Harrison *et al* 2001 and 2002, www.nci.nih.gov].

2.2.1.7. Diagnosis

The first diagnostic test is usually the blood count where leukaemic blasts are in most cases (but not all) detected. The diagnosis of ALL is based on the finding in bone marrow aspiration and biopsy. Apart from the classical morphology and cytochemistry, the bone marrow sample is investigated by immunophenotyping, cytogenetics and molecular biology methods. Further examinations enable the evaluation of the disease extent (cerebrospinal fluid sampling, chest X-ray, abdominal ultrasound) and possible other disorders and complications (blood coagulation and biochemistry, others).

During the ongoing treatment, multiple tests are carried out to evaluate the disease response, toxicity of the treatment and other complications. The disease response is assessed according to the presence or amount of blasts in peripheral blood and bone marrow at certain time-points, and recently by the presence and level of minimal residual disease (MRD) [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Harrison *et al* 2001 and 2002, www.nci.nih.gov].

2.2.1.8. Treatment

Treatment of childhood leukaemia should be carried out at specialised medical centres with all up-to-date facilities, by multidisciplinary teams of specialists with large experience in paediatric haematology, intensive and supporting care. The therapy of childhood ALL is coordinated on at national and international levels using designed treatment protocols that follow the state of art in paediatric haematology and evidence-based medicine principles and ensure the best outcomes. In general there are two options: the treatment according to standard protocols and treatment within clinical trials. The standard protocols incorporate the treatment that has been proven to be the most effective and is currently accepted as standard and recommendable. The clinical trials are very precisely and unambiguously designed studies that evaluate new, potentially better drugs or treatment approaches and compare them with the standard therapy. They are absolutely necessary for progress in the disease management. The participation in these trials should be offered to all eligible patients, their enrolment into the study requires an inform consent of the family.

The treatment of childhood ALL is assigned according to the risk groups defined by both clinical and laboratory findings (Table 2.9.). This stratified risk-based therapy ensures patients with excellent prognosis are not overtreated with possible long-term side effects and patients with poor prognosis receive the most intensive treatment from the beginning, which should offer the best achievable results.

The therapy consists of several phases: induction, consolidation or intensification, re-induction and maintenance therapy.

Induction includes the application of corticosteroids (dexamethasone or prednisone) and vincristine, combined with other cytostatics like asparaginase and daunorubicin. The combined chemotherapy may be

preceded by one week of application of prednisone alone. The duration of induction is 4 – 12 weeks and its aim is to achieve morphological remission with < 5 % of blasts in bone marrow and eradication of the extramedullary disease.

Table 2.9. Childhood ALL risk groups.

CCG (Children's Cancer Group) ALL risk groups	
standard risk	age 1 –9 years and WBC < 50 x 10^9/l at diagnosis
high risk	all other patients
very high risk	t(9;22) translocation or hypodiploidy (DNA index < 0.95) or M3 marrow at D29, M2-3 marrow at D43

POG (Pediatric Oncology Group) ALL risk groups	
low risk	age 1 –9 years and WBC < 50 x 10^9/l at diagnosis and favourable cytogenetics (either t(12;21)(TEL–AML1) translocation or trisomy of 4 or 10) and absence of adverse translocations and absence of CNS and testicular disease
standard risk	all other patients
high risk	age < 1 or > 9 years or WBC > 50 x 10^9/l at diagnosis or translocations with MLL gene rearrangement or absence of favourable cytogenetics with CNS or testicular disease
very high risk	t(9;22) translocation or hypodiploidy (DNA index < 0.95) or M3 marrow at D29, M2-3 marrow at D43
T-ALL are treated together with lymphoblastic T-cell NHL according to specific protocols	

BFM (Berlin-Frankfurt-Münster) ALL risk groups	
standard risk	age 1 – 5 years and WBC < 20 x 10^9/l at diagnosis and B-ALL immunophenotype and good initial prednisone response
medium risk	all other patients
high risk	t(9;22) translocation (or BCR-ABL fusion) or t(4;11) translocation (or MLL-AF4 fusion) or poor initial prednisone response or no CR at D33

The post-induction treatment incorporates further intensive therapy in an effort to eradicate the residual leukaemic cells (minimal residual disease, MRD). This consists of consolidation or intensification during the weeks 12 to 24, followed by re-induction (late intensification) during weeks 24 - 32. For consolidation, the protocols tend to add drugs different from the induction phase to avoid cross resistance, most often including methotrexate, 6-mercaptopurine, cyclophosphamide, cytarabine and adriamycine; for re-induction the same combination like for induction is often used.

Most protocols continue the treatment with maintenance therapy. The classical maintenance therapy consists of oral 6-mercaptopurine and methotrexate; monthly pulses of intravenous vincristine and oral corticosteroids may be either added to this combination or may replace it in certain protocols. This phase lasts till the end of the year 2 – 3 and its aim is to maintain remission and prevent relapse.

In addition to the systemic chemotherapy, the treatment of ALL includes also CNS directed therapy, prophylactive or curative. This is directed to eradicate the either subclinical or overt leukaemic infiltration of CNS, which in case of ALL is very frequent. The CNS-directed therapy includes intrathecal administration of methotrexate alone or together with cytarabine and hydrocortisone, combined with systemic administration of methotrexate. In case of proved CNS infiltration and high-risk groups, radiotherapy of CNS is added (cranial or craniospinal radiotherapy, 12 - 18 Gy).

The groups of patients with the highest risk of relapse are indicated for bone marrow transplantation in first complete remission [Degos *et al* 2000, Knowles *et al* 2001, Otten *et al* 2002, Berman *et al* 2000, Harrison *et al* 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000 and 2001, Ravindranath *et al* 2003, Schrappe *et al* 2002, www.nci.nih.gov].

New promising treatment options are molecular therapy and immunotherapy, at present tested in clinical trials with encouraging results (see 2.2.1.11.)

Apart from the antileukaemic therapy itself, the complex treatment of leukaemia includes extensive supportive therapy. It involves the prevention and treatment of symptoms and complications caused either by the disease or by antileukaemic therapy, thus enabling the administration of the antileukaemic treatment in full doses and within the recommended periods of time and preventing fatal complications. The supportive care includes application of blood derivatives, antimicrobial drugs, growth factors, analgesics, other different drugs according to the individual problems, fluids and nutrition, and physical and psychological rehabilitation.

2.2.1.9. Relapse

In the absolute majority of childhood ALL cases, the initial antileukaemic therapy manages to achieve the complete response, first complete remission (CR). However, a proportion of the patients consequently relapse, with recurrence of the disease. The relapse rate in childhood ALL is around 30 % and prognosis of these patients is unfavourable. Most of them attain a second remission but the likelihood of cure is generally poor. The relapse may occur at any time, during the ongoing treatment or after its completion. An early relapse appears either during the front-line therapy or within the first six months of its discontinuation and has very poor prognosis. A late relapse appears after 30 months of complete remission duration, usually responds well to the chemotherapy and its prognosis is more favourable. The

relapse is predominantly encountered in the bone marrow, but up to 30 % of relapses affect extramedullar sites, particularly CNS and testes, either together with the bone marrow involvement or as the only site of relapse manifestation (isolated extramedullary relapse occurs in up to 10 % of cases). Relapse is thought to be caused by leukaemic cells persisting in the body despite all the previous antileukaemic therapy. The phenotypic and cytogenetic characteristics of the leukaemic blasts at relapse are usually identical to those seen at diagnosis, but in small percentage of cases they differ, suggesting a clonal evolution. Relapse must be distinguished from a secondary leukaemia, a newly occurred leukaemia arising as a consequence of mutagenic effect of antitumour therapy applied for a previous malignancy. As the current ALL treatment protocols avoid the use of cytostatics with high risk of the secondary leukaemia development, the occurrence of secondary leukaemia after ALL treatment is rare [Degos *et al* 2000, Knowles *et al* 2001, Otten *et al* 2002, Berman *et al* 2000, Harrison *et al* 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, www.nci.nih.gov].

The treatment of relapse starts with induction chemotherapy employing similar drugs and combinations as in the first line induction. Using this approach, about 85 % of patients achieve second complete remission. At this stage, an early relapse is a definitive indication for allogeneic bone marrow transplantation, which may be also considered in the other patients. In case of late relapse, intensification with autologous bone marrow transplantation may be an effective and safer option. CNS relapse is treated with intrathecal chemotherapy combined, if possible, with craniospinal radiotherapy (depending on previous applied doses of radiotherapy) and with systemic application of high-dose methotrexate and cytarabine. Testicular relapse is treated like bone marrow relapse with the addition of radiotherapy to both testes [Degos *et al* 2000, Knowles *et al* 2001, Otten *et al* 2002, Berman *et al* 2000, Harrison *et al* 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, www.nci.nih.gov].

2.2.1.10. Bone marrow transplantation

Bone marrow transplantation (BMT) or peripheral stem/progenitor cell transplantation (PSCT/PBPCT) is an approach of intensified antileukaemic therapy. Following a conditioning regimen that consists of high-dose chemotherapy and/or total body irradiation the patient receives haematopoietic stem cells (by a short transfusion) obtained either from the bone marrow or peripheral blood of a donor. The transplantations are divided into two principle types according to the relation between the donor and the patient: allogeneic and autologous. In case of allogeneic transplantation, the donor is either a HLA-matched sibling donor (MSD), a HLA-matched unrelated donor (MUD) or a HLA-haploidentical relative donor. In case of autologous transplantation the donor is the patient himself, from whom the stem cells are taken usually during remission. The two main sources of stem cells, bone marrow and peripheral blood show partially different characteristics and each has some advantages and disadvantages. The bone marrow stem cells are obtained by several aspirations of bone marrow, usually from the pelvic bones. The peripheral stem cells are obtained from the peripheral blood by cytapheresis after mobilisation of stem cells. Normally, only very few stem cells and other haematopoietic progenitors are present in the circulating peripheral blood but they may be released from the bone marrow into the peripheral blood in

substantially higher amounts following certain stimulation. This stimulation is called mobilisation and in practice is achieved by the use of chemotherapy, growth factors or a combination of both. Currently, growth factors either alone or following chemotherapy are used for this purpose. After growth factor application, not only stem cells but also more mature progenitor cells are recruited and are present in the collected graft. This is probably responsible for the faster recovery of myelopoiesis and trombopoiesis after PSCT/PBPCT in comparison with BMT. In the case of autologous transplantation, the possible contamination of the graft with malignant cells is also lower when using peripheral stem/progenitor cells than bone marrow. On the other hand, in some donors, especially patients after many previous cycles of chemotherapy, mobilisation may be problematic and in these cases BMT is the only option. The collected bone marrow or peripheral stem cells may be manipulated before their administration to the patient (purging in case of autologous transplantation, T-cell depletion in case of allogeneic transplantation, gene transfer in experimental studies). In paediatric patients, another important source of stem cells with special characteristics may be used, the umbilical cord blood stem cells.

There are several factors that contribute to the therapeutic effect of allogeneic BMT or PSCT. The conditioning regimen is administered to achieve two objectives: antileukaemic effect and immunosuppression. The intensive highly dose-escalated chemotherapy or radiotherapy is supposed to be able to eradicate those leukaemic cells resistant to standard treatment. In addition to this, the conditioning must be sufficiently immunosuppressive to prevent rejection of the transplanted donors stem cells (allograft). The transplanted stem cells have two main roles: to replace the patient's normal stem cells destroyed by the intensive conditioning regimen and restore haematopoiesis, and to realise the graft-versus-leukaemia (GVL) effect, which is a special type of graft-versus-host (GVH) reaction. The GVL effect currently seems to be more important for the curative effect of BMT or PSCT than the high-dose conditioning regimen. Autologous PSCT or BMT lack GVL, which is probably the main reason for their considerably lower effectiveness, rather then possible contamination of the graft with malignant cells. The understanding of the importance of GVL has led to the introduction of non-myeloablative allogeneic BMT or PSCT, also called miniallotransplantations, where the conditioning regimen is aimed to have only immunosuppressive effect ensuring engraftment of the donor's stem cells and development of GVL with the antileukaemic effect. This approach significantly reduces the peritransplant morbidity and mortality associated with allogeneic transplantations while keeping most of their effectiveness. The non-myeloablative allotransplantations have shown to be beneficial in the therapy of various haematological malignancies but at present they are not involved in standard ALL treatment.

The criteria for allogeneic BMT indication in childhood ALL are still a matter of debate. The definitive indications for BMT from a matched sibling donor (MSD) are infant leukaemia, high-risk ALL in the first complete remission (CR1), and ALL in second complete remission (CR2) following an early bone marrow relapse (up to six months after completing the therapy). Bone marrow relapse after the first six months and an early CNS relapse are also indications where MSD BMT may be considered. High-risk patients in CR1 and patients in CR2 after an early bone marrow relapse are indicated for BMT from a matched unrelated donor (MUD) if there is not a MSD, the later are also considered for haploidentical BMT if a MUD is not available [Degos *et al* 2000, Knowles *et al* 2001, Otten *et al* 2002, Berman *et al*

2000, Harrison *et al* 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, Goulden *et al* 2002, www.nci.nih.gov].

2.2.1.11. Molecular therapy and immunotherapy

Studies of different molecular structures and pathways provide a molecular basis and potential targets for development of new therapeutic strategies. Many new substances and drugs, designed to affect the molecular mechanisms of leukaemogenesis, currently undergo pre-clinical testing. Some of them have already shown their efficiency in clinical trials and become a component of the complex antileukaemic therapy.

The main importance of the molecular approach is in their possible effect in those leukaemia cases or stages where conventional therapy, including chemotherapy, radiotherapy and stem cell transplantation, is not successful and curative. This therapy may be more specific than conventional modalities and therefore associated with lower side effects.

The new therapeutic strategies interfere with the leukaemogenic mechanisms on several levels: gene expression, signalling pathways and immune response.

Gene therapy involves methods that change gene expression by introducing of a gene into the cells of patients that brings therapeutic benefit. This is achieved by transferring nucleic acids with special coding sequences to the targets cells, using various methods and vectors that mediate successful transfer. The inserted genetic information may function in different ways. The genetic defect of malignant cells may be corrected by several techniques (ribozymes, antisense oligonucleotides, small interfering RNAs - siRNA, micro RNAs - miRNA, etc.) that block expression of the oncogene products. Malignant cells may be selectively destroyed, either directly or indirectly by decreasing their resistance to other modes of therapy, by so called suicide gene transfer (for example genes encoding enzymes converting non-toxic pro-drugs to highly toxic cytostatic agents). On the contrary, the sensitivity of normal cells, particularly haematopoietic stem cells, to the toxicity of antileukaemic treatment, may be modified by transfection with genes increasing their resistance (for example multidrug resistance gene MDR-1). Genetic immunomodulation may enhance the antitumour immune response by introducing genes that increase the immunogenicity of malignant cells (like genes encoding MHC antigens) or that facilitate the host immune response (like cytokines encoding genes).

A number of new drugs interfere with signalling pathways, abnormally activated in malignant cells. They inhibit pathological, deregulated and constitutive activation of signal proteins involved in accelerated proliferation and suppressed differentiation, and induce apoptosis of leukaemic cells (for example tyrosine kinase inhibitors, serine/theronine kinase inhibitors, transcription factors modulators, cell-cycle inhibitors, inductors of apoptosis, proteasome inhibitors, chromatin remodelling agents – hypomethylating agents and histone deacetylase inhibitors, telomerase inhibitors, inhibitors of MDR1, inhibitors of angiogenesis) [Chang *et al* 2003, Kim *et al* 2003, Ravandi *et al* 2002].

Immunotherapy is based on targeting specific, tumour-associated antigens on the malignant cells. The antibody therapy consists of the administration of monoclonal antibodies that specifically bind to the leukaemic antigens. Their antileukaemic effect results from blocking molecules that are important for

functions or interactions of malignant cells, or from the mediated cytotoxic effect (antibody-dependent cellular cytotoxicity mechanism). The antibodies may also be conjugated with other cytotoxic substances, like cytostatics or radioisotopes, which ensures their selective effect on the malignant cells. The antigens on malignant cells may represent targets for specific cytotoxic T-lymphocytes, stimulated against them, which is the principle of the immunoadoptive therapy and antitumour vaccines [Degos *et al* 2000, Ravandi *et al* 2002, Hoelzer *et al* 2002, Berman *et al* 2000, Griffin *et al* 2001, Perentesis *et al* 2001]

Tyrosine kinase inhibitors

Imatinib mesylate, STI571, is a 2-phenylaminopyrimidine derivate and a selective tyrosine kinase inhibitor (STI). It inhibits all Abl kinases including BCR-ABL and TEL-ABL kinase activity, all PDGFβ-R tyrosine kinases including TEL-PDGF-R, Arg and c-Kit. The mechanism of its action is based on blocking the binding site for ATP in the kinase. This prevents the ability of the kinase to transfer phosphate groups from ATP and phosphorylate tyrosine residues on the substrate proteins, thus inhibiting the activation of the downstream signal molecules and interrupting the signal transduction.

Because of its strong selective effect on BCR-ABL fusion protein activity, imatinib is a novel treatment option for leukaemia with BRC-ABL rearrangement. The clinical feasibility and efficacy of imatinib therapy was first proven in CML. In ALL with BCR-ABL rearrangement, which represents an ALL subgroup with very unfavourable prognosis, phase II trials showed a significant effect of imatinib in relapsed or refractory patients and phase II trials in newly diagnosed, previously untreated BCR-ABL positive ALL patients are ongoing.

Unfortunately, especially in patients with acute leukaemia, a resistance to imatinib may frequently develop during the treatment with this agent. The molecular mechanisms behind this cellular resistance are the subject of intensive research and several possible treatment strategies to overcome or prevent the resistance problem have been proposed. The resistance to imatinib seems to be multifactorial. The main causes of the resistance identified to date include amplification of BCR-ABL gene and specific point mutations (substitutions at several distinct sites) in the catalytic domain of BCR-ABL kinase. Overexpression of the multidrug resistance P-glycoprotein was also detected in certain cells with BCR-ABL rearrangement. Binding of imatinib to the drug-binding plasma protein α_1-acid glycoprotein is another possible, though controversial cause of resistance. In addition, suboptimal inhibition of BCR-ABL *in vivo* by imatinib could contribute to the selection of resistant cells. Combination of imatinib with other compounds targeting various downstream signal transduction pathways (like PI3K or Jak inhibitors), with conventional antileukaemic drugs or with nuclear export inhibitors may be a promising approach to increase the efficiency of imatinib and overcoming the problem of resistance. In a CML cell line model, it was shown that imatinib facilitates the transport of the BCR-ABL fusion protein from cytoplasm to the nucleus. A subsequent treatment with a nuclear entrapper leptomycin leads to nuclear accumulation of the chimeric protein inactivated by imatinib without affecting the cell survival. However, reactivation of the tyrosine kinase activity of the trapped BCR-ABL after imatinib treatment removal results in rapid apoptosis of these cells. Most importantly, combination therapy of imatinib with leptomycin shows significant synergism in inducing leukaemic cell death [Hernandez-Boluda *et al* 2002,

Gambacorti-Passerini *et al* 2003, Anastasiadou *et al* 2003, Ravandi *et al* 2002, Ravindranath *et al* 2003, Schrappe *et al* 2002].

Many other tyrosine kinase inhibitors with different specificities have been found and are tested at present, including Hsp90 inhibitors, interaction blockers, phospatases, etc. [Ravandi *et al* 2002, Hoelzer *et al* 2002, Berman *et al* 2000, Kelly *et al* 2002, Anastasiadou *et al* 2003].

Farnesyl transferase inhibitors

Farnesyl transferase inhibitors block the postranslational modification of Ras that involves the C-terminal farnesylation catalysed by farnesyl transferase. This modification is necessary for Ras association with the cellular membrane and its function. Farnesyl transferase inhibitors inhibit farnesylation and thus function of both normal and mutated Ras, however in malignancies with pathologically activated Ras their effect is more pronounced. Tipifarnib is a representative of these drugs, recently tested in clinical studies.

Similar drugs, geranylgeranyl (prenyl) transferase inhibitors have been successfully tried in clinical trials [Ravandi *et al* 2002, Hoelzer *et al* 2002, Clark *et al* 2003].

Antibody therapy

Contrary to some other haematological malignancies, where antibody therapy is an established part of the treatment, this approach in acute leukaemia still remains experimental. Rituximab (monoclonal anti-CD20) could have a potential role in B-ALL expressing CD20 (which is rather rare in childhood ALL), and require evaluation in further studies. Other monoclonal antibodies, like anti-CD19 combined with genistein or PAP and anti-CD22 in B-ALL, anti-CD52 and anti-CD7-ricin in T-ALL, have been investigated in pilot trials [Degos *et al* 2000, Ravandi *et al* 2002, Hoelzer *et al* 2002, Berman *et al* 2000, Countouriotis *et al* 2002, Clark *et al* 2003].

2.2.1.12. Outcome

The prognosis of ALL has considerably improved in the last 20 years as a result of more precise diagnosis enabling an assessment of the risk, an intensive treatment modulated according to the risk groups, introduction of new drugs and new modes of therapy and large improvements in supporting care. The 5-year overall survival (OS) is 75 – 85 % and the 10-year OS about 70 % [Degos *et al* 2000, Knowles *et al* 2001, Otten *et al* 2002, Berman *et al* 2000, Harrison *et al* 2001 and 2002, Hoelzer *et al* 2002, Greaves *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, www.nci.nih.gov].

2.2.1.13. Late consequences

The progress in the therapy of ALL has significantly improved cure rates. With the increasing number of long-term surviving ALL childhood patients, the question of the late side effects and complications of the antileukaemic treatment has become of high importance.

Chemotherapy, radiotherapy and allogeneic BMT may cause late side effects of several types: disorders of physical and mental development, infertility, impairment of organs or organ systems, and

induction of secondary malignancies. These complications may develop many years after the completion of the therapy. Most of them are dose-dependent and many also age- and sex-dependent (more pronounced at younger age, post-radiation CNS effects more pronounced in girls). As the therapy of ALL has changed progressively during the past 20 – 30 years, the incidence and pattern of the long-term effects of antileukaemic treatment has also been changing accordingly. Based on results of clinical trials, some components of ALL therapy protocols with proven high rate of serious late toxicities have been eliminated, reduced in doses or reserved for high-risk patients, without compromising the treatment outcome (e.g. chemotherapy combinations containing alkylating agents, topoisomerase II inhibitors and anthracyclines, radiotherapy of CNS). On the other hand, the introduction of new intensive protocols that significantly increase the survival of patients with unfavourable prognosis (e.g. bone marrow transplantation) brings new types of delayed effects.

The most common late effects of antileukaemic therapy are growth retardation (caused by hypothalamic-pituitary axis disturbance and growth hormone deficiency), cognitive dysfunctions and deficits (caused by radiation and/or chemotherapy induced white matter damage), neuroendocrine dysfunctions and infertility (caused either by hypothalamic-pituitary axis disturbance or gonadal damage), organ impairment - cardiomyopathy, neuropathy, disorders of other organs, avascular bone necrosis, and longer-term immunosuppression. Allogeneic bone marrow transplantation is often complicated by chronic graft-versus-host disease (GVHD) with possible manifestation in many different organs or body sites. The most serious long-term consequence of ALL therapy is the risk of development of a secondary malignancy. The overall cumulative risk of a second neoplasm is about 2 - 3 % in 15 years from diagnosis. These secondary malignant tumours are thought to be caused by both radiotherapy and chemotherapy (particularly alkylating agents and topoisomerase II inhibitors), in combination with predisposing genetic factors (in patients with secondary brain tumours after ALL therapy a genetic defect of thiopurine methyltransferase (TPMT) has been described leading to significantly higher levels of thioguanine nucleotide metabolites in blood). CNS tumours are the most common secondary malignancy, followed by other solid tumours (sarcomas, thyroid tumours, others) and secondary AML [Degos *et al* 2000, Knowles *et al* 2001, Shusterman *et al* 2000, Neglia *et al* 1991, Baker *et al* 2003, Bhatia *et al* 2002].

2.2.2. Acute myeloid leukaemia in childhood

AML in children and adults have more in common than any other respective paediatric and adult neoplasms. Therefore the main approach to childhood and adult AML is very similar and also many clinical trials group adult and childhood AML together.

2.2.2.1. Demography

Acute myeloid leukaemia is less frequent than ALL, comprising about 15 % of childhood acute leukaemias, but still belongs among the first ten most common paediatric malignancies. The incidence of AML in European countries and North America is around 0.5 – 0.6 / 100 000 and is relatively stable during last decades. The ratio of childhood ALL to AML is about 5 : 1. The incidence is higher in infancy

up to the age of 1 year, then falls down and in children between 1 – 10 years is low. Since adolescence it permanently increases throughout the life. There are no significant differences in the incidence of AML between different genders, except in infants, where there is female predominance. There are geographic differences in the incidence of AML, which is considerably higher in Asian countries [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, www.nci.nih.gov].

2.2.2.2. Etiology and risk factors

There is more known about the etiology of AML than ALL. Like in ALL, the malignant transformation may occur prenatally or during the postnatal life. Several groups of risk factors predisposing to and possibly inducing the development of AML have been identified (Table 2.10.). AML may arise *de novo* or develop from a preleukaemic conditions, aplastic anemia and myelodysplastic syndrome (MDS).

Monozygotic twin affected by AML in infancy, during the first year of life, brings about 20 % risk of developing AML also in the other sibling. The cause is supposed to be a common leukaemogenic mutation event in the early zygotic period or transmission of the malignant cells via a shared placental blood supply in the prenatal period. Children with Down's syndrome comprise 10 % of paediatric AML (the ratio between ALL and AML in Down's syndrome is approx. 2 : 1), and in most cases it is leukaemia of megakaryoblastic type. Several other genetic diseases are associated with a higher risk of MDS and/or AML development; children with neurofibromatosis 1 (NF1) are also at increased risk of developing juvenile myelomonocytic leukaemia (JMML).

The major identifiable cause of AML in children is previous therapy for other malignancy, leading to the development of secondary, therapy-related AML [Stamatoyannopoulos *et al* 2001, Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, Ravindranath *et al* 2003, www.nci.nih.gov].

2.2.2.3. Pathogenesis

AML, like ALL, is a clonal disease, arising from one original clonal precursor or a small clone of related precursors with identical genetic characteristics. The precursor undergoes a series of genetic changes (chromosomal rearrangements, gene mutations, amplifications or deletions) leading to inactivation of tumour suppressor genes and activation of proto-oncogenes and gradual transformation of the normal cells into abnormal cells with dysplastic features and malignant cells.

2.2.2.4. Classification

Morphological classification

Diagnosis of AML is based on the presence of more than 30 % blasts in the bone marrow. The remission is defined by the number of blasts in the bone marrow under 5 %.

The FAB (French-American-British Cooperative Group) morphological classification of AML is the standard nomenclature. According to the blast morphology and cytochemistry AML is divided into eight main subtypes, M0 – M7 (Table 2.11.). The subtypes recall the derivation of the leukaemic blasts from undifferentiated precursors (M0) and lineage-restricted granulocytic (M1, M2, M3, M3v), monocytic (M4, M4eo, M5, M5a, M5b), erythroid (M6) or megakaryoblastic (M7) precursors. The AML subtypes most common in children are M1, M2, M4 and M5, each comprising around 20 % (15 – 30 %) of childhood AML cases. The remaining subtypes are rare, each forming around 5 % of childhood AML. In young children up to 2 years of age M4 and M5 subtypes predominate and form 80 % of all AML at this age.

Table 2.10. Risk factors of childhood AML.

Risk factors of childhood AML:	
genetic diseases	Down's syndrome D-trisomy 18 trisomy XYY syndrome Fanconi's anemia Schwachman's syndrome Kostmann's syndrome treated with G-CSF neurofibromatosis (NF-1) Bloom's syndrome Blackfan-Diamond's syndrome TAR syndrome affected monozygotic twin sibling
inherited non-genetic conditions	previous history of maternal fetal loss pre- or perinatal exposure to marijuana, alcohol, cigarettes
acquired conditions	aplastic anaemia myelodysplastic syndrome paroxysmal nocturnal haemoglobinuria
therapy-related AML (t-AML)	alkylating agent induced topoisomerase II inhibitor induced
external factors - physical - chemical	radiation – prenatal, postnatal electromagnetic field (?) benzene environmental mutagenes (pesticides, solvents)

Very rare types of AML are eosinophilic and basophilic leukemia. Another rare type with special characteristics is juvenile myelomonocytic leukemia (JMML, previously called juvenile chronic myeloid leukaemia) [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, www.nci.nih.gov].

Table 2.11. FAB classification of AML.

FAB classification of AML							
subtype	morphology	cytochemistry					
		MPO	SB	PAS	NSE	NAF	BU
MO	myeloblastic, without differentiation	–	–	–	–	–	–
M1	myeloblastic, without maturation	< 3 %	+	–	+	–	–
M2	myeloblastic, with maturation	> 3 %	+	–	+	–	–
M3	promyelocytic (APL)	+	+	–	+	–	–
M3v	microgranular variant of APL	+	+	–	+	–	–
M4	myelomonocytic	+	+	±	+	+	+
M4eo	myelomonocytic with abnormal eosinophils	+	+	±	+	+	+
M5	monocytic	–	–	±	±	+	+
M5a	monoblastic, without differentiation	–	–	±	±	+	+
M5b	promonocytic, with differentiation	–	–	±	±	+	+
M6	erythroblastic	–	–	+	–	–	–
M7	megakaryoblastic	–	–	±	±	±	–

SB – Sudan black B, MPO – myeoperoxidase, PAS – periodic acid Schiff, NSE – nonspecific esterase, NAF – α-naphtyl acetate esterase, BU - α-naphtyl butyrase; blood and urinary lysozyme is + in M4 and M5

Immunological classification

The leukaemic blasts express different antigens of haematopoietic cells. Over 90 % cases of paediatric AML express myeloid-related antigens (CD 3, 15, 33, w65, 11b, 36, HLA-DR). Lymphoid-related antigens of B-cells (CD10, 19, 20, 22, 24) and T/NK-cells (CD2, 3, 4, 5, 7, 56) may be expressed in 10 – 20 % and 20 – 40 % respectively. AML with monocytic differentiation expresses antigens of monocytic lineage (CD14), erythroblastic AML expresses erythroid antigens (glycoprotein A - GPA), and megakaryoblastic AML expresses antigens of megakaryoblastic lineage (CD41, 42, 61) (Table 2.12.). Certain antigen combinations correlate with morphology and karyotype [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, www.nci.nih.gov].

Table 2.12. AML immunophenotypes.

FAB subtype	immunophenotype	cytogenetics
M0, M1	CD13, CD33	del(7), del(7q), del(5), del(5q)
M2	CD19, CD56 or CD2, CD7	t(8;21) no specific translocation
M3, M3v	CD33, CD13, CD15; CD34–, HLA-DR–	t(15;17)
M4, M4eo, M5, M5a, M5b	CD14	t(11q23)
M6	GPA	
M7	CD41, CD42, CD61, CD65	

Cytogenetic and molecular classification

Cytogenetic or molecular abnormalities are detectable in about 80 % of childhood AML cases. The distribution of different chromosomal aberrations varies with age.

Like in ALL, the chromosomal alterations may be either numerical or structural. Numerical alterations include hypo- and hyperdiploidy and are less common in childhood AML than ALL. Structural abnormalities involve some of those found also in childhood ALL and some typical and unique for AML and its FAB type (Table 2.13.). The most important molecular abnormality is Flt-3 mutation, present in about 20 % of childhood AML [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, www.nci.nih.gov].

2.2.2.5. Prognostic factors

The prognostic factors, identified as significant independent predictors of AML outcome, involve a number of clinical and laboratory disease characteristics. These factors undergo constant change reflecting the changes and progress in AML diagnosis and therapy. Some factors have been proven and are generally accepted to be associated with unfavourable or favourable prognosis, while some remain controversial with different results across clinical studies. (Table 2.14.) [Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, www.nci.nih.gov].

In contrast to ALL, there is no generally agreed group stratification according to prognostic factors in AML.

2.2.2.6. Manifestation and course of the disease

AML presents like other acute leukaemias with symptoms of bone marrow failure and, less frequently, also extramedullary infiltration. Isolated masses of leukaemic infiltrates are often referred to as chloromas or granulocytic sarcomas.

In addition to the symptoms common to most acute leukaemias (signs of anemia - fatigue, pallor, event. dyspnoea; neutropenia - fever, stomatitis, infections; trombocytopenia - spontaneous bruises,

purpura, bleeding; and extramedullary spread - lymphadenopathy, splenomegaly, hepatomegaly, skeletal pain, symptoms of CNS involvement or testicle infiltration), certain FAB AML subtypes have further characteristic clinical presentations. Acute promyelocytic leukaemia (APL, FAB M3 or M3v) usually manifests with profound haemorrhagic diathesis as a result of disseminated intravascular coagulopathy (DIC). Gingival hypertrophy is often present in monoblastic leukaemias, chloromas occur in M2 with t(8;21) aberration and also in monoblastic leukaemias. Extramedullary leukaemia, especially skin infiltration (leukaemia cutis), is more common in infants.

The course of an untreated AML is characterised by a fast progression. Infection or haemorrhage are the most common causes of death also in AML [Hoffbrand *et al* 1999 and 2001, Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, www.nci.nih.gov].

Table 2.13. Structural chromosomal alterations in childhood AML.

Structural chromosomal alterations in childhood AML		
Chromosomal alteration	Gene(s) involved	Clinical characteristics
t(8;21)(q22;q22)	ETO–AML1	M2, mostly older children favourable prognosis
t(15;17)(q22;q21) t(11;17)(q23;q21)	PML-RARA PLZF-RARA	M3 and M3v associated with DIC
inv(16)(p13;q22) t(16;16)(p13;q22)	MYH11-CBFB	M4eo associated with CNS disease favourable prognosis
t(9;11)(p22;q23)	MLL–AF9	M4, M5 mostly infants and younger children associated with high WBC and CNS disease
t(6;9)(p23;q34)	DEK-CAN	M2, M4
del(5), del(5q)		M0, M1 mostly older children
del (7), del(7q)	NF-1 and oth.	M0, M1, JMML mostly older children
t(1;22)		M7

2.2.2.7. Diagnosis

Diagnostic tests are similar to those in ALL. If extramedullary chloroma is the initial AML manifestation, its biopsy may also lead to the diagnosis. Further tests are carried out during the therapy aimed at evaluation of the disease response, toxicity of the treatment and other complications.

Table 2.14. Prognostic factors in childhood AML.

High risk prognostic factors in AML	
clinical presentation - leukocyte count	 WBC > 20 - 100 x 10^9/l at diagnosis
biological characteristics - inducing factors - cytogenetic and molecular aberrations	 secondary AML (t-AML) del(7), del(7q) Flt-3 internal tandem duplication
treatment response - BM blasts at the end of induction - MRD at the end of induction	 induction failure (no CR after 1 cycle of induction th.)
Low risk prognostic factors in AML	
biological characteristics - cytogenetic and molecular aberrations	 Down's syndrome chromosomal alterations t(15;17), t(8;21), inv(16)
treatment response	early response (CR after 1 cycle of induction th.)
Controversial prognostic factors in AML	
patient characteristics - age	 infants < 1 y
clinical presentation - disease development - organ involvement	 MDS presentation CNS leukaemia at diagnosis
biological characteristics - FAB type - immunophenotype - cytogenetic and molecular aberrations	 M4, M5, M7 CD34+, MDR (P-glycoprotein) expression 8 trisomy chromosomal alterations t(9;11), t(11q23)

2.2.2.8. Treatment

As stated previously, the treatment of childhood acute leukaemia should be carried out at specialised centres, by teams of experienced specialists and according to the approved standard protocols or within controlled clinical trials.

There is no generally agreed risk group stratification in childhood AML and patients are mostly treated according to common protocols with the exception of patients with APL (M3 AML), whose therapy is different.

The therapy of AML consists of two main phases: induction, and postinduction (consolidation and intensification, eventually maintenance therapy).

Induction consists of application of cytarabine and anthracyclin, either daunorubicin or recently rather idarubicin. Other agents like etoposide or 6-thioguanine may be added to this basic combination. With these regimens 80 – 85 % patients achieve complete remission (CR). The induction usually lasts 4 – 6 weeks and its aim is to achieve morphological remission with < 5 % of blasts in bone marrow and eradicate clinically detectable extramedullary disease. A profound bone marrow aplasia following the induction chemotherapy is considered a necessary prerequisite for remission induction. As this may be

connected with considerable morbidity and mortality, an intensive supportive care at this phase is a crucial component of the therapy.

The post-induction, or post-remission, treatment includes further intensive therapy that should eliminate the residual leukaemic cells (MRD) and sustain the remission. For consolidation, administered during following 4 – 6 weeks, the same cytostatics as for induction are used in combination with other agents including corticosteroides, cyclophosphamide, L-asparaginase, vincristine, fludarabine. This is followed by intensification during next 2 – 5 months. If a HLA-matched sibling donor (MSD) is available, allogeneic BMT is the option of choice at this stage of therapy. If there is no MSD, intensification with a regimen of high-dose cytarabine plus another cytostatic drug or autologous BMT are considered. Children with Down's syndrome have a better response to the chemotherapy than other patients and can be cured by chemotherapy alone. BMT is therefore not indicated in these children in the first remission.

Except APL, maintenance therapy is not generally recommended. When it is employed, it is usually used in those patients who were not indicated for BMT and consists of administration of 6-thioguanine and cytarabine of duration up to 1 year. Recently, immunomodulatory therapy with interleukin-2 following completion of consolidation phase is under evaluation in clinical trials.

CNS-directed therapy is now incorporated into most childhood AML treatment protocols. It is administered in prophylactive or therapeutic settings and consists of intrathecal application of cytarabine or cranial radiotherapy alone or in combination [Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Greaves *et al* 2002, Ravindranath *et al* 2003, www.nci.nih.gov].

Acute promyelocytic leukaemia (APL, M3) with t(15;17) translocation has unique characteristics and its treatment differs from other AML subtypes. It is sensitive and responds well to the treatment with all-trans-retinoic acid (ATRA), which is the principal component of APL treatment regimens. In induction and consolidation phases, ATRA is combined either with antracyclines alone or cytarabine plus anthracycline. Prophylactic intrathecal chemotherapy and intensification with BMT are not indicated but maintenance therapy with ATRA alone or with other cytostatics is administered. Under evaluation is another agent with an important activity in APL, arsenic trioxide. In adult patients it has shown interesting results in clinical trials; data for children are rather limited so far. An uncommon variant of APL with t(11;17) translocation is much less sensitive to ATRA treatment and its therapy may follow common protocols for the other AML subtypes [Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Greaves *et al* 2002, Ravindranath *et al* 2003, www.nci.nih.gov].

The intensive antileukaemic therapy can be realized only with concomitant maximal supportive therapy.

2.2.2.9. Relapse and refractory AML

About 15 % of children with AML do not achieve remission with the induction therapy and up to 50 % of patients relapse from remission during the first 5 years, over half of these relapses occurring within one year after diagnosis. In most cases the relapse occurs in the bone marrow, extramedullary relapses are very rare. The length of the first remission is the most important predictor of the probability to attain a

second remission; CR1 duration less than 1 year is connected with very poor prognosis. Regimens based on high-dose cytarabine in combinations with other agents (mitoxantron, L-asparaginase, anthracyclines, others) and therapy with fludarabine and 2-chlorodeoxyadenosine may introduce a second remission in more than 50 % of the patients but this sustains only in 20 % of them or less. BMT performed in second remission may cure 20 – 40 % of selected patients with recurrent or refractory disease. MRD BMT is preferred, in case of unavailable matched related donor match unrelated donor, haploidentical related donor, cord blood stem cell or autologous BMT may be considered. Experimental therapy is tested in clinical trials (e.g. monoclonal antibodies against CD33 or 45). Recurrent APL may be treated with regimens containing ATRA or arsenic trioxide with about 80 % patients achieving second remission. t-AML have in general very poor prognosis, those with 11q23 chromosomal aberrations show better initial response but cure is infrequent [Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, Ravindranath *et al* 2003, www.nci.nih.gov].

2.2.2.10. Molecular therapy and immunotherapy

Like in ALL, molecular therapy and immunotherapy represent new very promising approaches also in the treatment of AML [van der Velden *et al* 2003, Degos *et al* 2000, Ravandi *et al* 2002, Berman *et al* 2000, Countouriotis *et al* 2002, Clark *et al* 2003, Larson *et al* 2003]. The therapy with imatinib mesylate, STI571, can be effective in rare cases of AML with BCR-ABL gene fusion or in AML with c-Kit mutations, but there are not enough clinical data available to evaluate the role of imatinib role in these leukaemia subtypes. Several promising tyrosine kinase inhibitors have been tried in AML with Flt-3 mutations. It is of importance that different inhibitors show distinct effects against different types of Flt-3 mutations (ITD, point mutations in codon 835 and 836) [Grundler *et al* 2003, O'Farrell *et al* 2003, Tse *et al* 2002, Yee *et al* 2002, Levis *et al* 2002, Anastasiadou *et al* 2003]. HDAC inhibitors have been tested in APL with the aim of reversing the transcriptional inhibition of RARα fusion proteins in recruiting HDAC and other corepressors [Grimwade *et al* 2002].

Immunotherapy with monoclonal antibodies, particularly anti-CD33, alone or in conjugation with calicheamicin or I^{131}, and anti-CD45, has been undergoing initial clinical trials [van der Velden *et al* 2003, Ravandi *et al* 2002, Berman *et al* 2000, Countouriotis *et al* 2002, Lo-Coco *et al* 2003a].

2.2.2.11. Outcome

Although there has been a great improvement in the therapy of AML achieved during past 20 years, the treatment outcomes are still not satisfying. The overall survival is about 45 – 50 % in 5 years.

Long-term surviving patients may suffer from the late side-effects of the antileukaemic treatment similar to those after treatment for ALL. The incidence of secondary AML (which may be sometimes difficult to distinguish from relapse) is higher as a result of using more mutagenic cytostatics in AML therapy regimens [Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, Ravindranath *et al* 2003, www.nci.nih.gov].

2.2.2.12. Juvenile myelomonocytic leukaemia

Juvenile myelomonocytic leukaemia (JMML) is a special type of childhood leukaemia accounting for less than 1 % of childhood leukaemia cases. This disease possesses myeloproliferative features and sometimes is rather classified as chronic leukaemia, though it has no relation to true CML. It typically presents in young children, more commonly in boys, and is relatively frequent in children with neurofibromatosis and chromosome 7 monosomy. The diagnosis is suggested by clinical criteria (hepatosplenomegaly, lymphadenopathy, pallor, fever, skin rash) and based on laboratory criteria (Table 2.15.).

The prognosis of JMML is poor and, despite improvements in the therapy of JMML, the overall survival in 5 years remains low. JMML patients should be treated within clinical trials, the protocols employ chemotherapy similar to that of AML protocols, BMT, ATRA and experimental drugs (e.g. farnesyl transferase inhibitors) [Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, Greaves *et al* 2002, Ravindranath *et al* 2003, www.nci.nih.gov].

Table 2.15. Juvenile myelomonocytic leukaemia diagnostic criteria.

JMML diagnostic criteria	
minimal laboratory criteria (all have to be fulfilled)	- no Ph chromosome, no BCR-ABL rearrangement - PB monocytes > 1 x 10^9 / l - BM blasts < 20 %
further laboratory criteria (≥ 2 have to be fulfilled)	- Hb F > normal for the age - myeloid precursors in PB - WBC > 10 x 10^9 / l - clonal abnormality (incl. 7 monosomy) - in vitro GM-CSF hypersensitivity of myeloid progenitors

2.2.3. Hybrid acute leukaemia in childhood

Hybrid acute leukaemia is a rare kind of acute leukaemia where leukaemic blasts posses both lymphoid and myeloid features. They could be present in the same cell in the case of biphenotypic acute leukaemia (BAL), or on separate cell populations in the case of bilineal acute leukaemia. The definition of BAL is based on a scoring system (Table 2.16.) [Hoffbrand *et al* 1999, Degos *et al* 2000, www.nci.nih.gov].

2.2.4. Acute undifferentiated leukaemia in childhood

Acute undifferentiated leukaemia (AUL) is a very rare kind of acute leukaemia in which the leukaemic blasts are highly undifferentiated with features of primitive progenitor cells, stem cells. The

cells show no evidence of differentiation and do not express any morphologic, immunophenotypic, genotypic, genomic or functional characteristic of either lymphoid or myeloid lineage [Testa *et al* 2002].

Table 2.16. BAL scoring system.

BAL scoring system			
points	B-lineage	T-lineage	My-lineage
2	CD79a cCD22 cIgμ	c/sCD3 TCR α/β TCR γ/δ	MPO (lysozyme)
1	CD19 CD10 CD20	CD2 CD5 CD8 CD10	CD13 CD33 CDw65
0.5	TdT CD24	TdT CD7 CD1a	CD14 CD15 CD69 CD117
BAL: score > 1 for lymphoid lineage and > 2 for myeloid lineage			

(c- cytoplasmic, s - surface)

2.2.5. Secondary therapy-related acute leukaemia

Secondary therapy-related acute leukaemia is a rare but often fatal consequence of previous therapy for a malignant disease. The risk of a secondary malignancy, among which secondary leukaemia is one of the most frequent, is the most serious late side effect of antitumour treatment. Both chemotherapy and radiotherapy have potential direct mutagenic and carcinogenic effects. The development of secondary malignancy may be further supported by prolonged immunosuppression, which is another common side effect of antitumour therapy. It also seems that interindividual differences in metabolism of cytostatic agents, like variable activity of NAD(P)H quinone oxidoreductase (HQO1) or glutathione-S-transferase, may be responsible for the different individual susceptibility to these agents. And finally, there are probably other genetic predispositions in those patients developing t-AML, although cases with germ-line tumour suppressor gene mutations, like p53, Rb, or WT1, are exceptional.

The secondary leukaemia or therapy-related acute leukaemia is AML in more than 90 % of cases (t-AML), but therapy-related ALL may also occur. There are two distinct forms of t-AML: those associated with alkylating agents and those associated with topoisomerase II inhibitors.

t-AML following alkylating agent therapy is more common. It emerges around 5 years (2 – 10 years) after the treatment, is often preceded by a myelodysplastic syndrome (MDS), is poorly differentiated

(M0/M1 type) and has characteristic cytogenetic abnormalities - del(7), del(7q) , del(5) or del(5q). The risk of alkylating agent-induced t-AML is dose-dependent and potentiated by radiotherapy and splenectomy. The mechanism involved in its development may include a specific susceptibility to chromosome breakage at the centromere, hence the increased frequency of dicentric chromosomes and the loss of whole chromosomes or their arms.

t-AML following therapy with inhibitors of DNA topoisomerase II, like epipodophyllotoxins or intercalating agents, has different characteristics. This type arises earlier after the treatment, 2 years on average (6 months – 5 years), usually does not have a preleukaemic myelodysplastic phase and is of more differentiated type (M4/M5). Chromosomal alterations typical for this leukaemia are translocations involving 11q23 with MLL gene rearrangements or similar, like 11p15 translocations involving NUP98 and 21q22 translocations involving AML1. All these alterations result from double-strand DNA breaks caused by topoisomerase II inhibitors and errors in their repair [Hoffbrand *et al* 1999, Degos *et al* 2000, Knowles *et al* 2001, Hall *et al* 2001, Grimwade *et al* 2001, www.nci.nih.gov].

2.2.6. Acute infant leukaemia

Infant leukaemia, diagnosed in the first 12 months of life, displays unique biological and clinical features and differs from the rest of acute childhood leukaemias. In this age group, the distribution of ALL and AML is equal and affected female infants slightly predominate over males. Most, if not all, cases of infant leukaemia arise *in utero*. Despite this very early onset it seems that the transmission of parental germinal mutations is quite a rare cause of infant leukaemia and in the majority of cases the leukaemogenic mutation or mutations are acquired during prenatal development.

The major consistent genetic abnormality, present in 60 – 70 % of infant leukaemia of both ALL and AML types, is the rearrangement of the MLL gene, located on chromosome 11 (11q23). This gene may be fused with many different partners in a number of chromosomal alterations, the most common of them is t(4;11) (AF4-MLL). There is increasing evidence that MLL gene mutations in infant leukaemia may be a result of prenatal exposure to topoisomerase II inhibitors due to an increased maternal consumption of food containing these agents. The individual susceptibility to the exposure to these agents may be influenced by parental variations in the activity of the enzymes metabolising topoisomerase II inhibiting agents like NAD(P)H quinone oxidoreductase (HQO1, NQO1).

In infant leukaemia cases lacking MLL gene rearrangements, a possible role of other carcinogenes and the parental genetic susceptibility factors involved in modulating the effect of parental carcinogen exposure has been suggested. For example, in parents of the affected infants, significantly higher frequencies of deletions of genes encoding glutathione-S-transferase, an enzyme antagonising the mutagenic effect of many carcinogenes and radiation, have been found.

The etiopathogenesis and biology of infant leukaemia thus resemble in many aspects the treatment-related leukaemia.

The presence of leukaemic clones at the time of birth has been detected in many cases with consequent development of childhood leukaemia. However, there is an increasing evidence that the postnatal immune

response and potential immune-mediated elimination of the leukaemic clones may play a major role in the eventual progress and manifestation of clinical disease.

Infant ALL has usually immature immunophenotype with lack of CD10 expression and frequent co-expression of myeloid-associated antigens. Infant AML is mostly of M4 or M5 subtypes. For both ALL and AML, high leukocyte count and extramedullary disease including CNS involvement are a typical presentation. Leukaemia in infancy is thus associated with multiple unfavourable prognostic factors, belongs to higher risk groups (in case of ALL) and generally has poor prognosis. Infant ALL is treated according to protocols especially designed for this age group. They can achieve 5-year disease-free survival in approximately 20 – 40 % of ALL patients. Infant AML is usually treated according to common protocols with similar results like in older children. Congenital AML in normal neonates occasionally regresses spontaneously but most require cytotoxic therapy. In neonates with Down's syndrome transient myeloproliferative syndrome (TMS), also called transient abnormal myelopoiesis (TAM), resembling M7 AML may occur. It recovers spontaneously within 4 – 6 weeks, however these children may later develop true AML [Hoffbrand *et al* 1999, Degos *et al* 2000, Biondi *et al* 2000a, Hall *et al* 2001, Ravindranath *et al* 2002, www.nci.nih.gov].

2.3. Minimal residual disease

The current treatment of childhood acute leukaemia, particularly ALL, is based on stratified and individualised approach, reflecting the patient's risks and disease prognosis (risk directed treatment). The risk/prognostic and predictive factors, which enable assessment of the probable disease course and response to the treatment, include patients' characteristics at diagnosis (age, sex, race), disease characteristics at diagnosis (leukocyte count, organ involvement, cytogenetic and molecular abnormalities) and early response to the initial therapy (peripheral blood morphology, bone marrow morphology, and recently minimal residual disease) [Moppett *et al* 2003, Degos *et al* 2000, Knowles *et al* 2001, Harrison *et al* 2001, Greaves *et al* 2002, Hoelzer *et al* 2002, Pui *et al* 2000, Ravindranath *et al* 2003, Cazzaniga *et al* 2002a and 2003].

2.3.1. Definition of minimal residual disease

Minimal residual disease (MRD) refers to the post-treatment presence of small submicroscopic amount of leukaemic cells in the bone marrow or other sites, undetectable by conventional morphological methods. Morphological bone marrow remission is defined by presence of less than 5 % of blasts in the bone marrow; the detection limit of cytomorphological techniques is 1 – 5 % of malignant cells. The amount of residual cells below this level represents the minimal residual disease (Fig. 2.14.) [Degos *et al* 2000, Campana *et al* 2003, Moppett *et al* 2003, Pui *et al* 2000].

At diagnosis, the total number of leukaemic cells in the body reaches 10^{11} - 10^{12}. When complete morphological remission is achieved by induction therapy, the amount of remaining leukaemic cells may vary within very broad ranges, in principal between 0 to 10^{10}. Rapid and profound decrease of the number of leukaemic cells may signalise the best chances of their total eradication leading to sustained complete remission, while slow reduction of the malignant cells by therapy, their persistence or reoccurrence may cause relapse of leukaemia. Detection of MRD enables discrimination and quantification of the residual leukaemic cells within levels $10^{4\text{-}6}$ - 10^{10}, thus offering a new insight into the disease course and treatment response.

2.3.2. Methods of minimal residual disease detection

Reliable methods of MRD detection should possess high sensitivity and specificity, ability to quantify, broad applicability, possibility of rapid result generation and easy standardisation. The sensitivity of the methods should reach at least 10^{-3} (one malignant cell among one thousand normal cells), ideally 10^{-4} to 10^{-6}. The high specificity means maximal discrimination between malignant and normal cells (low false-positivity) and, at the same time, detection of the maximal amount of malignant cells (low false-negativity). So far only two methods, immunophenotyping by flow cytometry and PCR techniques, meet the requirements and thus are used for MRD detection (Table 2.17.). Among other potential assays, FISH techniques show sensitivity 10^{-2} to 10^{-3} but their application is restricted to well-defined chromosome aberrations with available DNA probes; fluorescent genescan has also only limited sensitivity 10^{-2} to 10^{-3};

cell culture systems show variable sensitivity and their routine standardised application is difficult [Foroni *et al* 1999, Degos *et al* 2000, Campana *et al* 2003].

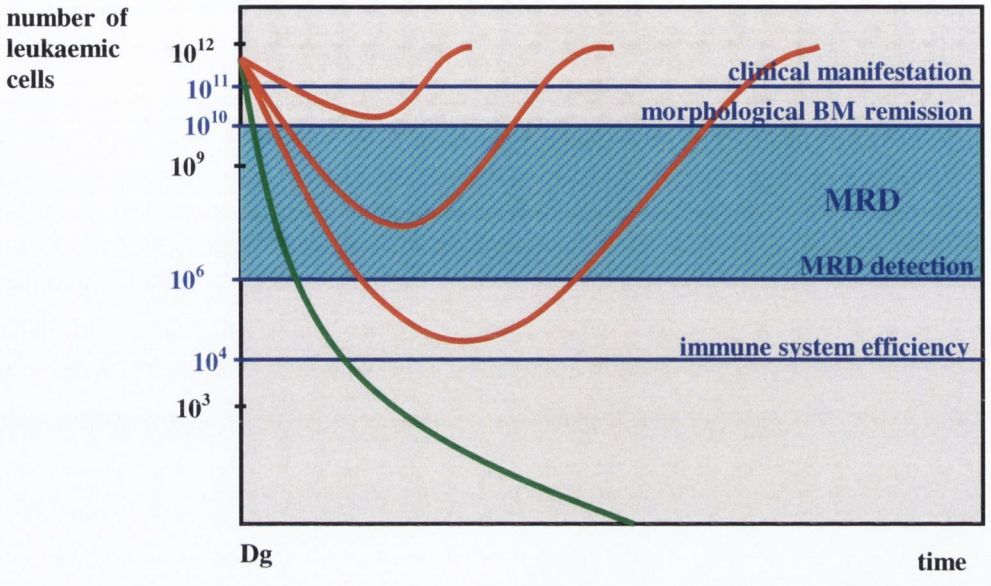


Figure 2.14. MRD and the kinetics of leukaemic cell levels during several possible courses of the disease; leading to permanent complete remission (green) or relapse (red).

Table 2.17. Methods of residual disease detection.

Methods of residual disease detection		
Method	Sensitivity	Remarks
Cytomorphology	5×10^{-2}	
Cytomorphology with cytochemistry	10^{-2}	
Cytogenetics	10^{-2}	Cells in metaphase only
FISH	$10^{-2} - 10^{-3}$	Well-defined chromosome aberrations only
Southern blotting	$10^{-1} - 10^{-2}$	Well-defined chromosome aberrations only
Fluorescent genescan	$10^{-2} - 10^{-3}$	Not standardised
Cell cultures	variable	Not standardised
Immunophenotyping by FC	$10^{-3} - 10^{-5}$	
Molecular biology - PCR of chromosomal aberrations - PCR of clonal IG/TCR rearrangements	$10^{-4} - 10^{-6}$	

2.3.2.1. Immunophenotyping by flow cytometry

Flow cytometry

Flow cytometry (FC) is a method that enables study and measurement of multiple intrinsic and evoked optical signals (light-scatter and fluorescence of labelling fluorochromes) from single cells or cell-sized particles in a moving fluid stream (Figure. 2.15.).

The basic principle of flow cytometry relies on using a fluidic system to deliver cells of a random suspension to a specific point intersected by the illuminating laser beam. The cells pass the laser beam one by one and reflect the light forward (forward scatter, FSC) or in right angle (side scatter, SSC) based on their properties (FSC depends on the cell size, SSC on the internal structure of the cell, in particular granularity). If the cells are labelled with fluorescent dyes, the laser beam of a certain wavelength causes excitation of the fluorochromes and emission of light of other specific wavelengths. The most commonly used fluorochromes are fluorescein isothiocyanide (FITC), R-phycoerythrin (PE, RPE), peridinin-chlorophyll protein (PerCP), allophycocyanin (APC), propidium iodide (PI), AlexaFluor, and fluorochrome conjugates like phycoerythrin-cyanine5, 5.5 or 7 (PE-Cy5, 5.5 or 7), allophycocyanin-cyanine7 (APC-Cy7) and others. Some of these fluorochromes are employed directly to label cellular components, like nucleic acids (eg. PI), but most of them are used to covalently label other probes, like monoclonal antibodies against cellular antigens. The application of fluorochrome conjugates is based on the phenomenon of fluorescence resonance energy transfer (FRET) where the light emitted by one fluorochrome leads to excitation of the other fluorochrome in the conjugate (the emission wavelength of one fluorochrome must correspond to the excitation wavelength of the other). The scatter and the fluorescent light generated by cells are collected by a system of photodetectors which convert the photon pulses into electric signals, and further electronic and computational processing results in graphic display and statistical analysis of the sample. The combination of FSC, SSC and use of several fluorochromes allows simultaneous measurement of multiple parameters on a single cell (eg. 6 parameters in case of 4-colour FC) [Ormerod *et al* 2000, Owens *et al* 2000, Campana *et al* 2000, 2002 and 2003, Bain *et al* 2002].

The system can also be used for cell sorting based on multiparameter analysis.

Detection of minimal residual disease by flow cytometry consists of the detection of leukaemia-associated phenotypes and detection of clonality.

Detection of leukaemia-associated phenotypes

Leukaemia-associated phenotypes (LAPs) are abnormal, aberrant, uncommon antigen expression patterns (AAE) of malignant cells, which do not occur in normal haematopoietic cells at all or are very rare (< 0.01 %). There are several causes and types of LAP expression (Table 2.18.).

Detection of LAPs enables MRD monitoring in 80 – 90 % of childhood B-ALL, over 95 % of childhood T-ALL and 70 – 90 % of AML, with sensitivity 10^{-3} – 10^{-5}.

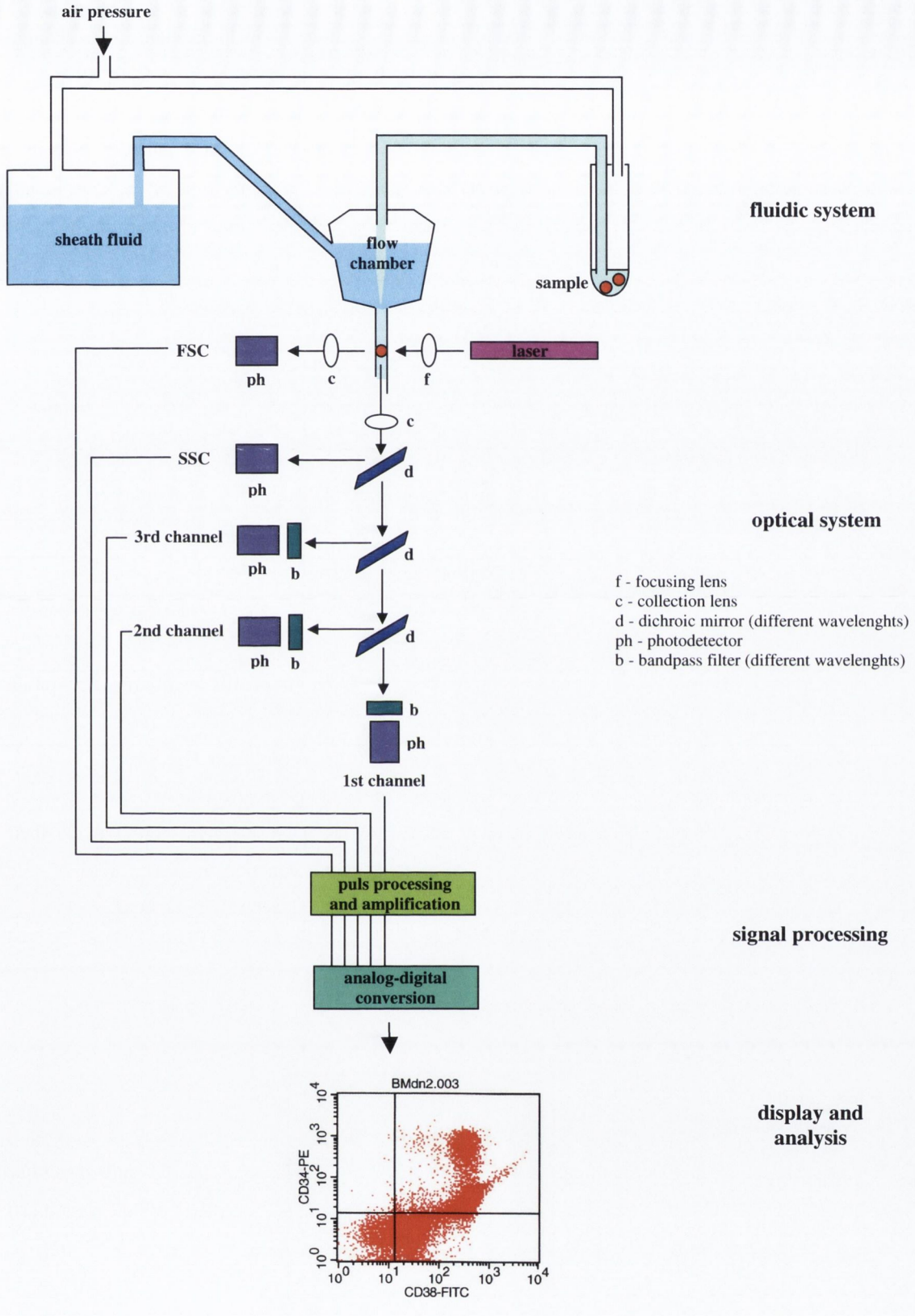


Figure 2.15. Scheme of flow cytometer.

A change in antigen expression pattern during course of the disease, in case of relapse or progression of leukaemia, may cause problems in MRD detection by FC and may possibly lead to false negative results. Excluding a secondary malignancy due to mutagenic therapy, the main mechanisms of changing antigen patterns are immunophenotypic shifts during clonal evolution, and lineage switches. Changes of at least one LAP during the disease course occur in up to 60 – 80 % of cases, and changes of several or all LAPs detected at diagnosis have been reported in up to 20 % of cases. In addition, quantitative changes in antigen expression during chemotherapy have been recently described. It is therefore necessary to identify several LAPs in one patient (≥ 2 – 3) and use all of them for MRD monitoring to minimise the risk of false negative findings [Degos *et al* 2000, Campana *et al* 1995, 1999, 2000, 2002 and 2003, San-Miguel *et al* 2002, Lucio *et al* 2001, Oelschlagel *et al* 2000, Baer *et al* 2001, Gaipa *et al* 2005].

Table 2.18. Leukaemia-associated phenotypes.

Leukaemia-associated phenotypes (LAPs):	
quantitative:	expression of normal Ag in abnormal intensity
Ag overexpression	expression of Ag higher than normal
Ag underexpression	expression of Ag lower than normal or none
qualitative:	expression of abnormal Ag or abnormal combinations of Ags
maturation asynchronous Ag expression	simultaneous expression of Ags of different maturation stage
cross-lineage Ag expression	co-expression of Ags of different lineages
ectopic Ag expression	expression of Ag not normally present - normal Ag of haematopoietic cells outside their normal sites of production or homing areas - normal Ag of non-haematopoietic cells - tumour-associated Ag

(Ag - antigen)

Detection of clonality

It is possible to detect clonal rearrangements of TCRα/β or γ/δ by FC using specific antibodies raised against the V domain of TCRβ, γ or δ, respectively. Because of the limited sensitivity of this method (10^{-2} – 10^{-3}), it is not at present used for MRD detection [Degos *et al* 2000, Campana *et al* 1999 and 2003].

2.3.2.2. PCR techniques

PCR and real-time quantitative PCR

The polymerase chain reaction (PCR) is a highly sensitive technique for gene expression detection. It consists of multiple cycles of template denaturation, primer annealing and extension (elongation), which results in amplification of DNA or cDNA sequences of interest, defined by the primers used. It is an exponential process that generates amplified product in amount enough to be visualised by electrophoresis on an ethidium bromide-stained agarose gel. As a template, DNA can be used directly, RNA has to be first converted into cDNA (complementary DNA) by reverse transcription (RT) (Figure 2.16.).

Real-time quantitative PCR (RQ-PCR) is a PCR-based method that enables quantification of the DNA or cDNA sequence of interest. It includes use of fluorescent dyes or fluorescent-labelled probes, which emit fluorescence during the reaction depending on the amount of the target present. The fluorescent signal is detected in real time and during the linear phase of the reaction it is directly proportional to the starting copy number of the target sequence and the PCR cycle. Different fluorescent probes and several RQ-PCR technologies (TaqMan, LightCycler, others) are currently available (Table 2.19.) TaqMan 5' nuclease assay with a dual-labelled probe is the most widely used RQ-PCR system so far. It employs a single sequence specific internal probe conjugated with a reporter fluorochrome (like 6-carboxyfluorescein, FAM) at its 5' end and with a quencher dye (like 6-carboxy-tetramethyl-rhodamine, TAMRA) at its 3' end. The proximity of the reporter to the quencher in an intact probe results in suppression of the reporter fluorescence. During the extension phase, this probe is hydrolyzed by the nuclease activity of the Taq polymerase. The cleavage of the probe separates the reporter from the quencher, which leads to increased fluorescence (Figure 2.17.) [Applied Biosystems manuals, Invitrogen manuals, Qiagen manuals, Cazzaniga *et al* 2003, van der Velden *et al* 2003, Neale *et al* 2003].

Two different PCR methods may be used for MRD detection, PCR of clonal IG/TCR rearrangements and PCR of chromosomal aberrations.

PCR detection of clonal immunoglobulin and T-cell receptor rearrangements

Variable domains of immunoglubulins (IGs) and T-cell receptors (TCRs), that are responsible for the unique antigen recognition, are formed during early lymphoid differentiation via gene rearrangement process. During this process, germ-line gene segments are randomly joined together and the joining ends are modified by random deletion of original and insertion of new nucleotides (see chapter 2.1.3.). Later in the development, these sequences may be further modified by somatic mutations and secondary rearrangements. The junctional regions of rearranged *IGs* and *TCRs* thus represent unique, fingerprint-like sequences, which are under normal conditions shared only by few lymphoid cells of the same clone. As acute leukaemia is a clonal disease, most or all cells of the leukaemic population possess these unique and characteristic sequences, which can be therefore used as a specific marker for MRD detection. These sequences are patient-specific. Contrary to normal lymphoid cell development, *IG* and *TCR*

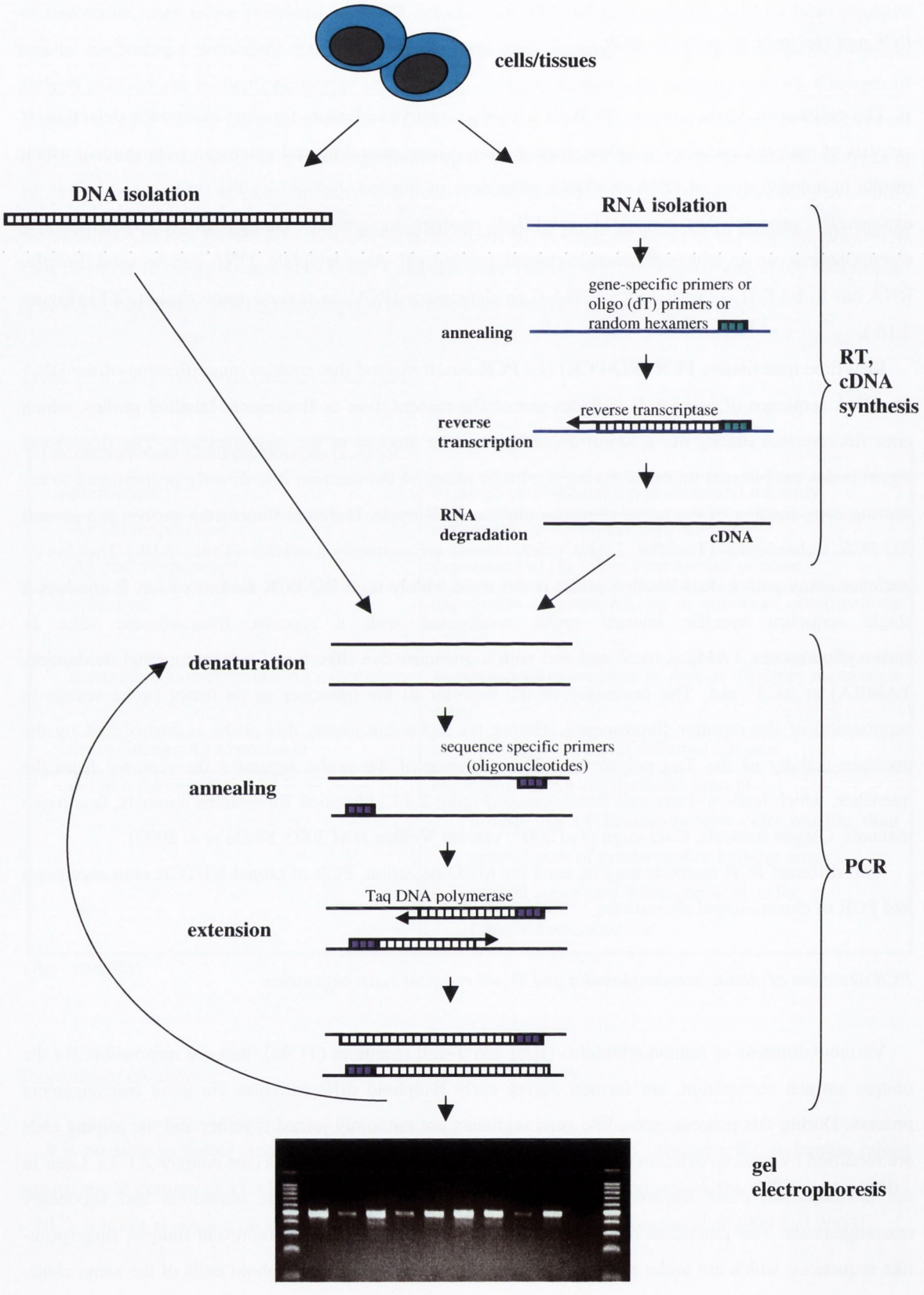


Figure 2.16. Scheme of RT and PCR reactions.

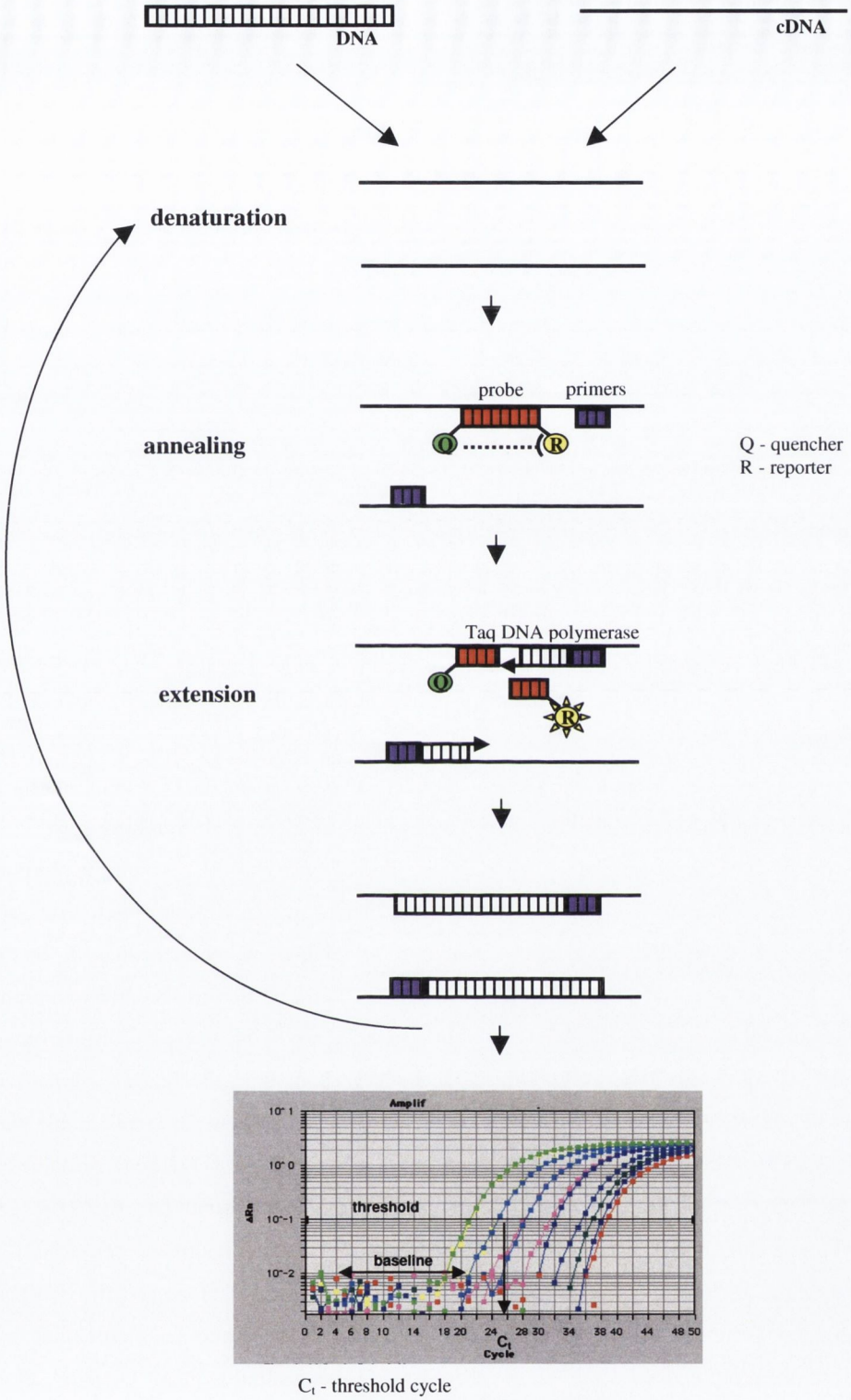


Figure 2.17. Scheme of RQ-PCR reaction.

Table 2.19. Real time PCR detection systems.

Real time PCR detection systems:	
dsDNA binding dyes	SYBR Green
fluorogenic probes - hydrolysis:	dual-labelled probes
- hybridisation:	FRET probes
- others:	molecular beacons
	scorpions
	minor groove-binding probes
	quantiprobes

rearrangements in haematological malignancies are not lineage-restricted and all of them may occur in B-ALL, T-ALL and AML.

PCR analysis of clonal *IG/TCR* gene rearrangements enables MRD detection in 90 – 95 % of childhood B-ALL and over 95 % of childhood T-ALL, with sensitivity 10^{-3} – 10^{-5} (Table 2.20.). Interestingly, *IG/TCR* clonal rearrangements may be detected in about 10 % of AML, but because of the relatively low frequency and the labour intensity of this method they are usually not used for MRD detection in AML.

Recently, fluorescent gene scan has been used for detection of clonal *IG/TCR* gene rearrangements. This technique has a limited sensitivity (10^{-2} – 10^{-3}) but is relatively rapid and straightforward.

In some cases of acute leukaemia (20 – 40 %, mostly B-ALL, rarely T-ALL), multiple rearrangements leading to bi- or oligoclonality can be found (they have to be distinguished from so called pseudoclonality, caused by the presence of reactive, non-malignant lymphocytes in the sample). Oligoclonality together with subclone formation and clonal evolution due to secondary rearrangements and somatic mutations may complicate MRD monitoring and be a possible cause of false-negative results during the disease follow-up. The probability of secondary changes in the rearranged patterns is, in general, higher in the *IGH* gene than in the *TCR* genes. D - J rearrangements are more stable than V- DJ joint regions. *IG-Kde* rearrangements are recognised as the most stable MRD targets (preserved in 90 % of cases), the least stable are incomplete *TCRD* rearrangements (around 60 %). *TCR* genes are significantly more stable in T-ALL than B-ALL. However, a recent study [Germano *et al* 2003] found no major difference in stability of these genes and thus no preferential clone-specific MRD marker. Monoclonal targets are characterised by higher stability (up to 90 %), whereas oligoclonal targets are frequently lost at relapse (only 40 % preserved). Significant subsequent changes of *IG/TCR* rearrangements detected at diagnosis may occur in up to 20 % of cases and the rate increases with time. Use of 2 or 3 target sequences, preferentially representing 2 - 3 different gene loci, is therefore necessary for MRD monitoring to minimise the risk of false negative results. With the current possibilities for *IG/TCR* analysis, at least 2 independent MRD targets can be identified in approximately 90 % of B-ALL and T-ALL patients [Pongers-Willemse *et al* 1999, van der Velden *et al* 2002a, b, c, 2003 and 2004b,

Szczepanski *et al* 2000, 2001b, 2002a and b, Li *et al* 2001, Cazzaniga *et al* 2002a and 2005, van Dongen *et al* 2003, Germano *et al* 2003, Boeckx *et al* 2002, Pine *et al* 2003, Brueggemann *et al* 2004].

Table 2.20. Occurrence of IG and TCR rearrangements in childhood ALL.

Occurrence of IG and TCR rearrangements in childhood B- and T-ALL		
Rearrangement	B-ALL	T-ALL
IGH	95 %	-
- complete (VH-DJH)	80 %	
- incomplete (DH-JH)	15 %	
IGK	60 %	-
TCRG	55 %	90 %
TCRD	40 %	60 %
TCRB		90 %
TAL1 del	-	10 %

PCR detection of chromosomal aberrations

Chromosomal aberrations (translocations, deletions, amplifications, inversions) are present in a proportion of acute leukaemias. They give rise to tumour-specific fusion genes, many of them being directly involved in the process of oncogenesis. The breakpoint fusion regions of these chromosomal aberrations represent a convenient MRD marker. They are disease-specific, present in leukaemic cells, often characteristic for certain leukaemia types and subtypes, and never found in normal cells. They are also quite stable during the disease course. Certain chromosomal aberrations (with breakpoints within an area of up to 2 kb) may be diagnosed by PCR directly from DNA; the analysed sequence is then patient-specific. However, most of the chromosomal aberrations are detected by RT-PCR from fusion mRNA, which is the same common product of possible many distinct breakpoints within large intronic DNA regions. Here, the analysed sequence is not patient-specific, which brings some advantages (use of one standardised PCR reaction for all patients with the particular aberration) and disadvantages (risk of cross-contamination) (Table 2.21.).

PCR of breakpoint fusion regions of chromosomal aberrations enables MRD detection in 40 – 50 % of childhood B-ALL, 10 – 30 % of childhood T-ALL and 30 – 40 % of childhood AML, with sensitivity 10^{-4} – 10^{-6} [Degos *et al* 2000, van Dongen *et al* 1999, Campana *et al* 2003, van der Velden *et al* 2003 and 2004a, Cazzaniga *et al* 2002b, Gabert *et al* 2003, Beillard *et al* 2003].

Table 2.21. The most common chromosomal aberrations and their occurrence in childhood acute leukaemia.

The most common chromosomal aberrations in acute leukaemia.			
Chromosomal aberrations	B-ALL	T-ALL	AML
all	40 – 50 %	10 – 25 %	30 – 40 %
t(12;21) TEL-AML1	15 – 25 %		
t(1;19) E2A-PBX1	5 – 15 %		
t(4;11) MLL-AF4	5 %		
t(9;22) BCR-ABL p190 and p210	5 %		
microdel (1p) SIL-TAL1		10 – 25 %	
t(10;11) CALM-AF10		10 %	
t(15;17) PML-RARA			5 – 15 %
t(8;21) AML1-ETO			5 – 15 %
inv(16p) CBFB-MYH11			5 – 10 %

2.3.2.3. Other potential PCR markers

Many other gene and molecular abnormalities and markers, that are found in acute leukaemia, have been proposed and tested as targets for MRD detection (Table 2.22.) [van der Velden *et al* 2003, Campana *et al* 2003, Cazzaniga *et al* 2002a]. Probably the most interesting and promising of them is the overexpression of Wilms' tumour gene 1 (*WT1*), detected in high percentages (70 – 90 % of cases) in all acute leukaemia types [Cilloni *et al* 2002 and 2004, Kreuzer *et al* 2001, Menssen *et al* 2002, Trka *et al* 2002, Ogawa *et al* 2003]. The possible value of *FLT3* internal tandem duplications (ITD) in detection of MRD in AML is decreased by its unstable presence during the disease course, being undetectable at relapse in a proportion of patients [Schnittger *et al* 2002, Kottaridis *et al* 2002 and 2003, Stirewalt *et al* 2001, Lo Coco *et al* 2003b, Grimwade *et al* 2002, van der Velden *et al* 2003, Campana *et al* 2003, Cazzaniga *et al* 2002a].

New potential MRD markers are currently a subject of intensive research but so far, neither *WT1* overexpression nor other markers have been recommended and validated for a standard use in MRD monitoring.

The advent of microarrays that enables rapid analysis of global gene expression profiles of leukaemic and normal bone marrow cells has opened new opportunities to identify suitable markers for MRD detection.

Table 2.22. Further potential MRD markers.

Further potential MRD markers	
chromosomal aberrations:	t(9;11) MLL-AF9
	t(11;14) TTG2-TCRa/d
	t(5;14) HOX11L2
	itd13q12 Flt3
inactivation of tumour suppressor genes:	p16 and p15
	p53 and p73
	Rb
mutation of oncogenes:	Ras
abnormal expression of genes:	WT1, MDR1
alteration of apoptosis:	Bcl-2, apoptotic assays
alteration of proliferation:	telomerase/telomere length
distinct gene profile:	gene microarrays

2.3.2.4. Comparison of methods of MRD detection

Each of the methods of MRD detection has some advantages and disadvantages (Table 2.23.). In practice, very high correlation between the different methods of MRD detection has been shown by a number of studies [Neale *et al* 1999 and 2004, Chen *et al* 2001a, Malec *et al* 2001 and 2004, Munoz *et al* 2000, Goulden *et al* 2001, Gamiero *et al* 2002, Campana *et al* 2003, Kerst *et al* 2005].

The different methods of MRD detection may be employed together, either in tandem or consequently (eg. flow cytometric pre-sorting of leukaemic cells used then for further PCR tests, or flow cytometry analysis of fluorochrome-labelled PCR products), to increase their applicability, sensitivity and specificity [Neale *et al* 1999, Munoz *et al* 2000, Zhang *et al* 2002]. This approach however increases also the demand on equipment and staffing.

2.3.2.5. Detection of MRD in different tissue samples

Bone marrow is used for standard MRD detection. Apart from bone marrow, MRD may be detected in other tissues, like peripheral blood, cerebrospinal fluid, bone marrow or peripheral stem cell autograft.

In T-ALL, high concordance has been found in MRD levels between bone marrow and peripheral blood samples and peripheral blood could thus be used for MRD monitoring in T-ALL. In B-ALL, presence and level of MRD in peripheral blood are significantly lower than in bone marrow. Peripheral blood therefore cannot be used for MRD monitoring in B-ALL, but presence of MRD in peripheral blood identifies B-ALL patients who are at very high risk of relapse [Coustan-Smith *et al* 2002a, van der Velden *et al* 2002a, Szczepanski *et al* 2002a].

Techniques of MRD detection may enable early diagnosis of leukaemic CNS infiltration and confirm its clearance during therapy or indicate an early relapse [de Haas *et al* 2002, Nunez *et al* 2003].

Detection of residual leukaemic cells in bone marrow or peripheral stem cell autograft before transplantation can help to predict the outcome of the transplantation [Campana *et al* 2003, Cazzaniga *et al* 2003].

Table 2.23. Comparison of the standard methods of MRD detection.

Comparison of standard methods of MRD detection			
Characteristics	FC detection of LAPs	PCR of clonal IG/TCR rearrangements	PCR of chromosomal aberrations
applicability			
ch B-ALL	80 – 90 %	90 – 95 %	40 – 50 %
ch T-ALL	over 95 %	over 95 %	10 – 30 %
ch AML	70 – 90 %	(10 %)	30 – 40 %
sensitivity	$10^{-3} - 10^{-5}$	$10^{-3} - 10^{-5}$	$10^{-4} - 10^{-6}$
specificity	partially patient-specific	patient-specific	disease-specific
-specificity of MRD marker (discrimination of leukaemic from normal cells)	variable	high	high
-stability of MRD marker during disease course /relapse	75 – 80 %	75 – 80 %	high
-discrimination of viable from dying/dead cells	yes	no	no
quantification	absolute, accurate	relatively accurate	relative
practical implications			
-standardisation	possible	yes	yes
-need of special care and immediate processing of the sample due to instability of the target	yes	no	yes
-need of large amount of normal/ control samples	yes	no	no
-generation of results	rapid	slow	relatively rapid
-risk of cross-contamination	possible	no	yes
-demands on staff, equipment	moderate	high	moderate

2.3.2.6. Limitations of the methods of MRD detection

Despite the high accuracy of the methods of MRD detection, there are certain limitations which may influence the generated results. The sensitivity of all the methods is ultimately limited by the number of analysed cells. In practice, most of the tests screen only a minor fraction of all bone marrow cells, around $10^{-5} - 10^{-6}$, thus limiting the sensitivity to $10^{-4} - 10^{-6}$. Furthermore, the bone marrow sampling is often not fully representative because of possible heterogeneous distribution of the residual leukaemic blasts in bone marrow, especially in remission. Both of these factors could be responsible for possible false negativity. On the other hand, PCR-based assays cannot distinguish between viable and dying cells and none of the common methods of MRD detection can determine the self-renewal capacity of the leukaemic cells, leading to possible false positive results or overestimating of the residual leukaemic burden. Bone marrow regeneration after chemotherapy may, depending on the specificity of the MRD marker, increase the unspecific background or even lead to false positive results.

An increased sensitivity of the tests due to development and optimisation of the MRD detecting techniques together with analysing higher number of cells may give evidence of MRD presence at very low levels in high percentage of patients remaining in long-term complete remission. Here, further studies will be needed for adequate interpretation of such results.

Other possible causes of false positive or false negative results are non-standardised methodology, use of insufficient or inappropriate controls, inappropriate sample handling or laboratory techniques, or instability of the MRD marker due to clonal evolution.

For a routine implication of MRD detection in clinical practice, several important questions have yet to be answered: the sample (bone marrow, peripheral blood) and the number of cells analysed, the time points, and the appropriate parameters of the techniques used [Foroni *et al* 1999, Goulden *et al* 2001, Campana *et al* 2003, Cazzaniga *et al* 2003, van der Velden *et al* 2003].

Recently, an effort has been made to standardise the methods of MRD detection, to prevent some of the above problems and enable better comparison of results between different laboratories: BIOMED-1 and EAC reports on PCR of chromosomal aberrations [van Dongen *et al* 1999, Gabert *et al* 2003], BIOMED-1 and 2 reports on PCR of clonal *IG/TCR* rearrangements [Pongers-Willemse *et al* 1999, van Dongen *et al* 2003] and BIOMED-1 report on flow cytometric immunophenotyping in B-ALL [Lucio *et al* 2001].

2.3.3. Importance of minimal residual disease detection in childhood acute leukaemia

2.3.3.1. Theoretical considerations

At the time of diagnosis, the total number of leukaemic cells in the body reaches $10^{11} - 10^{12}$. During the initial treatment this number usually rapidly decreases and the vast majority of patients achieve complete remission with less then 1 – 5 % of blasts in bone marrow. However, this may represent a number of residual leukaemic cells in the body that is anything between zero and 10^{10}. Detection of MRD enables better and much more precise characterisation of the residual leukeamic burden persisting in the

patient's body at the time of morphologic bone marrow remission. The sensitivity threshold of the methods of MRD detection defines the molecular remission, which corresponds to a total number of leukaemic cells in the body of 10^{6-8} or less.

The presence, amount and kinetics of MRD have been proven to be one of the best indicators of the treatment response and overall outcome and thus one of the most important independent prognostic and predictive factors in acute leukaemia. Importantly, unlike other prognostic factors, MRD assessment has prognostic relevance also in the setting of relapse. High amounts of leukaemic cells, their slow decrease, persistence or reoccurrence after therapy indicate a disease refractory to the treatment, they are an obvious source of relapse and therefore represent a serious risk and prognostic factor. Also, the extent of reduction of leukaemic cells by the induction therapy clearly reflects their sensitivity to the treatment and the disease response and act as the best predictive factor.

MRD detection in acute leukaemia includes two principle objectives: evaluation of the initial response at the end of induction therapy and continuous monitoring during the remission. MRD evaluation at the end of induction may influence the following therapeutic strategy. It enables better stratification and very individual approach, which could improve the treatment outcomes. Intensification of therapy in high-risk patients may improve the cure rates, while reduction of the therapy in low-risk patients should prevent over-treatment and possible late side effects. Continuous monitoring during remission may detect the molecular relapse, defined as reappearance of detectable MRD in two successive BM samples. Molecular relapse, as well as increasing levels of MRD in patients who never achieved molecular remission, indicate a very high risk of the haematological relapse, and usually precede it by several weeks to months. Therapy started at the time of molecular relapse often induces better response and improves prognosis [Goulden *et al* 2001, Donadieu *et al* 2001, Campana *et al* 2003, van der Velden *et al* 2003, Moppett *et al* 2003, Cazzaniga *et al* 2003, de Haas *et al* 2001].

Specific applications of MRD detection involve detection of CNS leukaemia, detection of bone marrow involvement in case of extramedullary relapse of acute leukaemia, and detection of MRD in autologous BM or PBSC grafts.

2.3.3.2. Results of clinical studies

The clinical significance of MRD status evaluation and monitoring has been conclusively demonstrated by an increasing number of large-scale prospective clinical studies. Several studies also confirmed good concordance of results of different methods of MRD detection (Table 2.24.). However, there are discrepancies in results that can be observed across the studies, like differences in the most informative time points, the MRD cut-off values, the proportion of patients belonging to different risk groups based on MRD assessment and their risk of relapse. These differences could be related to a number of variables: different patients' and disease characteristics, different treatment protocols, different time points of MRD detection, different sensitivity and specificity of the methods of MRD detection and different controls employed, and different methods of statistical analysis of the data used in different studies. Consequently, the prognostic and predictive value of MRD detection is influenced by all these

variables [Degos *et al* 2000, van Dongen *et al* 1999 and 2003, Campana *et al* 1995, 2002 and 2004, Cazzaniga *et al* 2003, Hoelzer *et al* 2002, Donadieu *et al* 2001, Lucio *et al* 2001].

International clinical trials that include reassessment of the patients' risks and adjustment of the further treatment according the MRD status at the end of induction (day 28) and intensification (week 8 – 11) have been ongoing.

In childhood ALL the MRD presence and level at the end of induction therapy have been shown to be the most powerful predictive and prognostic factor, significant and independent, of outcome and risk of relapse. The most important time points appear to be around week 4 and week 12, possibly also day 15. Presence of MRD ($\geq 10^{-4}$, or even $< 10^{-4}$) is associated with higher risk of relapse, high levels of MRD ($\geq 10^{-2}$) being associated with particularly unfavourable prognosis [Cave *et al* 1998, van Dongen *et al* 1998, Coustan-Smith *et al* 2000 and 2002b, Panzer-Grumayer *et al* 2000, Pui *et al* 2001, Biondi *et al* 2000b, Willemse *et al* 2002, Dworzak *et al* 2002, Szczepanski *et al* 2001a and 2002a, van der Velden *et al* 2003, Campana *et al* 2002 and 2004, Cazzaniga *et al* 2002a and 2003]. Kinetics of the decrease in the leukaemic burden at two time points (eg. after induction and consolidation) may be more important than a single time point [van Dongen *et al* 1998]. The continuous monitoring during remission in ALL is of limited clinical value and significance to date. Steady decrease of MRD is usually associated with favourable prognosis while persistently high or steadily increasing levels of MRD signal high risk of relapse [Cave *et al* 1998, van Dongen *et al* 1998, Coustan-Smith *et al* 2002b and 2004, Szczepanski *et al* 2001a and 2002a, van der Velden *et al* 2003, Campana *et al* 2002 and 2004, Cazzaniga *et al* 2002a]. MRD status before bone marrow transplantation is a significant predictor of the outcome, no evidence of MRD being associated with good prognosis [Knechtli *et al* 1998a, Nagler *et al* 1999, van der Velden *et al* 2001, Goulden *et al* 2002 and 2003, Bader *et al* 2002]. Similarly, MRD presence or level after bone marrow transplantation is of clinical relevance [Knechtli *et al* 1998b, Goulden *et al* 2002 and 2003, Uzunel *et al* 2003]. MRD was shown to have a significant prognostic impact also in relapsed ALL [Eckert *et al* 2001, Coustan-Smith *et al* 2004].

In AML, the MRD evaluation at the end of induction and prospective monitoring after consolidation (for 6 – 12 months after consolidation) is particularly valuable in APL (M3 AML). Persisting PCR positivity after consolidation is associated with adverse prognosis and requires additional intensive therapy. Reoccurrence of PCR positivity (molecular relapse) precedes haematological relapse often by several months. The commencement of treatment at the time of molecular relapse has in APL a significantly better response and prognosis than when started at the time of haematological relapse [Burnett *et al* 1999, Grimwade *et al* 1999, 2001 and 2002, Lo-Coco *et al* 1999 and 2003b]. Pre- and post-transplant MRD monitoring is also valuable in patients undergoing allogeneic or autologous bone marrow transplantation for refractory or relapsed APL [Grimwade *et al* 2002]. In other subtypes of AML, the data are limited but, similar to ALL, suggest that evaluation of MRD at the end of induction is a powerful independent prognostic factor of relapse and outcome. Continuous monitoring in remission may be of clinical importance, high levels of MRD being associated with high risk of relapse and negative MRD status associated with durable remission [Laczika *et al* 2001, San-Miguel *et al* 2001 and 2002, Liu Yin *et al* 2002, Szczepanski *et al* 2001a, Venditti *et al* 2000, Coustan-Smith *et al* 2003]. MRD status before and after allogeneic bone marrow transplantation may also be of clinical relevance [Nagler *et al* 1999].

In case of autologous bone marrow or peripheral stem cell transplantation for both ALL and AML, MRD-positivity of the graft before purging is the most important predictive factor of the treatment failure, regardless of the efficiency of the purging procedure [Campana *et al* 2003, Cazzaniga *et al* 2003, van der Velden *et al* 2003, Venditti *et al* 2003].

MRD detection has many potential applications in the clinical management of patients with childhood acute leukaemia. In patients with slow clearance, persistence or re-emerging MRD levels, treatment intensification or reintroduction of therapy may improve the treatment outcome, decrease relapse rate and increase disease-free and overall survival. Conversely, patients with profound cytoreduction at the early stages of therapy and undetectable MRD levels may be cured with less intensive therapy that will spare them possible late side effects. However, it is important to realize that MRD negativity does not mean disease eradication and, on the other hand, low levels of MRD may be present in some patients staying in long-term complete remission. MRD-based treatment intervention studies that address the clinical benefit of using MRD for treatment stratification and relapse risk assessment in childhood acute leukaemia have been initiated and their first results should be available soon. Meanwhile, the main challenge is to simplify and standardise the methods of MRD detection so as they could be used in routine practice. Future MRD research has to also explore the biology of the residual leukaemic disease to understand the mechanisms and conditions of MRD persistence and recurrence [Campana *et al* 2003, Moppett *et al* 2003, Cazzaniga *et al* 2003].

Table 2.24. The most relevant published studies on detection of MRD in childhood ALL and AML - cont.

MRD detection in childhood ALL		
Author and year	Type of leukaemia	Method of MRD detection
Ciudad 1998	ALL	FC
Farahat 1998 (UK ALL)	ALL	FC
Griesinger 1999 (BFM)	ALL	FC
Coustan-Smith 2000 (St Jude)	ch ALL	FC
Coustan-Smith 2002 (St Jude)	ch ALL	FC
Dworzak 2002 (BFM)	ch ALL	FC
Bjorklund 2003 (NOPHO)	ch ALL	FC
Borowitz 2003 (POG)	ch pre-B ALL	FC
Cave 1998 (EORTC)	ch ALL	PCR IG/TCR
van Dongen 1998 (BFM)	ch ALL	PCR IG/TCR
Panzer-Grumayer 2000 (BFM)	ch ALL	PCR IG/TCR
Biondi 2000 (BFM)	ch B-ALL	PCR IG/TCR
Willemse 2002 (BFM)	ch ALL	PCR IG/TCR
Nyvold 2002 (NOPHO)	ch ALL	PCR IG/TCR
Scrideli 2002	ch ALL	PCR IG/TCR
Marshall 2003	ch ALL	PCR IG/TCR
Cimino 2000	ALL	PCR FG

Table 2.24. The most relevant published studies on detection of MRD in childhood ALL and AML.

Gameiro 2002 (MRC)	ch ALL	PCR IG/TCR, PCR FG
Malec 2001 (NOPHO)	ALL	FC, PCR IG/TCR
Neale 2004 (St Jude)	ch ALL	FC, PCR IG/TCR, PCR FG
Malec 2004 (NOPHO)	ch ALL	FC, PCR IG/TCR
Kerst 2005	ch ALL	FC, PCR IG/TCR
Eckert 2001 (BFM)	ch ALL relapse	PCR IG/TCR
Nagler 1999	ALL and AML posttransplant	FC
Sanchez 2002	ALL posttransplant	FC
Coustan-Smith 2004 (St Jude)	ch ALL relapse	FC
Knechtli 1998 (MRC)	ch ALL posttransplant	PCR Ig/TCR
Knechtli 1998 (MRC)	ch ALL pretransplant	PCR Ig/TCR
Uzunel 2001	ALL pretransplant	PCR Ig/TCR
Bader 2002	ch ALL pretransplant	PCR Ig/TCR
Uzunel 2003	ALL posttransplant	PCR Ig/TCR
MRD detection in childhood AML		
Author and year	Type of leukaemia	Method of MRD detection
Venditti 2000 (EORTC)	AML	FC
San Miguel 2001	AML	FC
Kern 2003	AML	FC
Sievers 2003 (CCG)	ch AML	FC
Coustan-Smith 2003 (St Jude)	ch AML	FC
Diverio 1998	AML	PCR FG
Grimwade 1999	AML	PCR FG
Lo Coco 1999	AML	PCR FG
Burnett 1999	AML	PCR FG
Morschhauser 2000	AML	PCR FG
Tobal 2000	AML	PCR FG
Marcucci 2001	AML	PCR FG
Guerrasio 2002	AML	PCR FG
Trka 2002 (BFM)	ch AML	FC, PCR WT1
Reichle 1999	AML posttransplant	FC
Venditti 2003 (EORTC)	AML pretransplant	FC

BFM - International Berlin-Frankfurt-Munster Study Group, POG - Pediatric Oncology Group, NOPHO - Nordic Society of Paediatric Haematology and Oncology, EORTC - European Organisation for Research and Treatment of Cancer, CCG - Children's Cancer Group

2.4. Wilms' tumour gene 1

2.4.1. WT1 gene

2.4.1.1. WT1 structure and functions

Wilms' tumour gene 1 (*WT1*, also *WIT2*), located on chromosome 11p13, encodes a zinc-finger transcription factor with an important role in oncogenesis. Alterations of *WT1* gene (mutations or overexpression) have been found in a number of malignancies and premalignant conditions.

This gene was first identified in Wilms tumour and is one of a group of genes associated with this malignancy (Table 2.25.). A mutation of both *WT1* alleles is a characteristic aberration found in some cases of Wilms' tumour, and mutations (translocations, small intragene deletions or insertions, substitutions) affecting *WT1* function were also found in other malignancies and pre-malignant syndromes, including several hereditary syndromes predisposing to leukaemia. On the other hand, overexpression of *WT1*, mostly unmutated, was found in 70 – 90 % of both ALL and AML, including childhood ALL and AML, in some CML cases and many other types of solid tumours [Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Scharnhorst *et al* 2001, www.nlm.nih.gov/OMIM].

Table 2.25. Group of genes associated with Wilms' tumour.

Genes associated with Wilms' tumour		
Gene	Location	Malignancies with the gene alteration
WT1 (WIT2)	11p13	Wilms' tumour, other solid tumours, leukaemia, premalignant syndromes
WT2 (MTACR1) (Multiple tumour-associated chromosome region 1)	11p15.5	Wilms' tumour, premalignant syndromes
WT3	16q	Wilms' tumour, premalignant syndromes
WT4 (FWT1) (Familial Wilms'tumour 1)	17q12-21	Wilms' tumour
WT5 (WTSL) (Wilms tumour, susceptibility to)	7p14-13	Wilms' tumour

WT1 family includes a number (at least 32) of WT1 protein isoforms, which are generated from the same DNA template. The *WT1* gene sequence consists of 10 exons, the flanking intron DNA and a multicomponent gene regulatory system including an autoregulatable 5' promoter (TATA-less and GC-rich promoter containing several SP1 binding sites), 5' and 3' enhancer elements and an intron 3 silencer [Fraizer *et al* 1994, Hirose *et al* 1999, Malik *et al* 2000]. The WT1 isoforms are results of alternative transcription initiation, alternative mRNA splicing, mRNA editing and alternative translation initiation (several AUG sites within exon 1 and alternative exon 1a – see below, and a non-AUG translation initiation site upstream and in-frame with the first AUG). They are localised in different nuclear or

subnuclear regions and show different, partially overlapping but also distinct functions and targets [Bruening *et al* 1996, Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Scharnhorst *et al* 2001, Wagner *et al* 2003]. An N-terminally truncated transcript was also described and the presence and activation of a cryptic promoter within *WT1* intron 5 is hypothesised [Dechsukhum *et al* 2000, Dumur *et al* 2002]. An alternative *WT1* transcript, *AWT1* (also short transcript *sWT1*), recently reported, maintains *WT1* exonic structure between exon 2 and 10 but deploys a new first exon located in intron 1 of *WT1* (exon 1a). This transcript does not contain the repression domain, otherwise it may exist in all splicing variants characteristic for *WT1* [Dallosso *et al* 2004, Hossain *et al* 2006].

Anti-sense *WT1* transcripts (*WT1-AS*) overlapping the 5' end of the *WT1* gene, with no apparent open reading frames, have been detected and a regulatory role of these RNAs suggested. The anti-sense promoter activated by WT1 has been identified in the first intron of *WT1* gene. *WT1-AS* is co-expressed with *WT1* [Moorwood *et al* 1999, Malik *et al* 2000].

Next 5' to the *WT1* gene on 11p13, there is a locus encoding another similar protein, WT1 associated protein (WIT1), transcribed in the opposite direction to *WT1*. The sequence in between the *WT1* and *WIT1* genes may act as a bi-directional promoter and multiple transcriptional start sites for both *WT1* and *WIT1* were identified in this region and the first exon and intron of *WT1*. *WIT1* expression in normal tissues resembles *WT1* but at lower levels. Also, a *WT1-AS/WIT1* fusion transcript containing sequences from both *WT1* and *WIT1* was described [Hofmann *et al* 1993, Campbell *et al* 1994, Scharnhorst *et al* 1999 and 2001, www.nlm.nih.gov/OMIM].

A related *WT2* gene is located on chromosome 11p15.5 and also linked to some hereditary syndromes with malignant predispositions.

The structure of WT1 proteins shows significant homology to EGR1 and 2 (early growth response factor-1 and 2). The polypeptide (about 500 amino acids) consists of an N-terminal proline-glutamine-rich transcriptional regulatory domains including transcription repression and transcription activation domains acting independently each other, RNA recognition domain and homodimerization domain; and C-terminal zinc finger DNA-binding domain containing four zinc fingers and two nuclear localisation signals. The zinc finger domain has been also demonstrated to bind to RNA. An alternative splicing generates four distinct transcripts which differ in the presence or absence of so called 17aa insert (17 amino acids encoded by the whole exon 5) and KTS insert (according to the 3 amino acids leucine-threonine-serine encoded by the terminal sequence of exon 9). The KTS insert is located between the third and fourth zinc fingers of the WT1 protein. Here, it alters the spacing between these zinc fingers, changing the DNA recognition site of the proteins, and so considerably influencing the WT1 protein function as well as its subcellular localisation. 17aa insert is located in the middle of the protein and may act as a transcriptional activation domain. The four major isoforms are called variants A [EX5-/KTS-], B [EX5+/KTS-], C [EX5-/KTS+] and D [EX5+/KTS+]. Isoforms containing both splice inserts are the prevalent variants, the least common transcripts are those missing both inserts. The conservation in structure and relative levels of the four main WT1 variants suggests that each of them makes a significant contribution to normal gene function [Renshaw *et al* 1997, Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Scharnhorst *et al* 2001, Hossain *et al* 2001, Moorwood *et al* 1999, Lee *et al* 2001, Dallosso *et al* 2004] (Figure 2.18.).

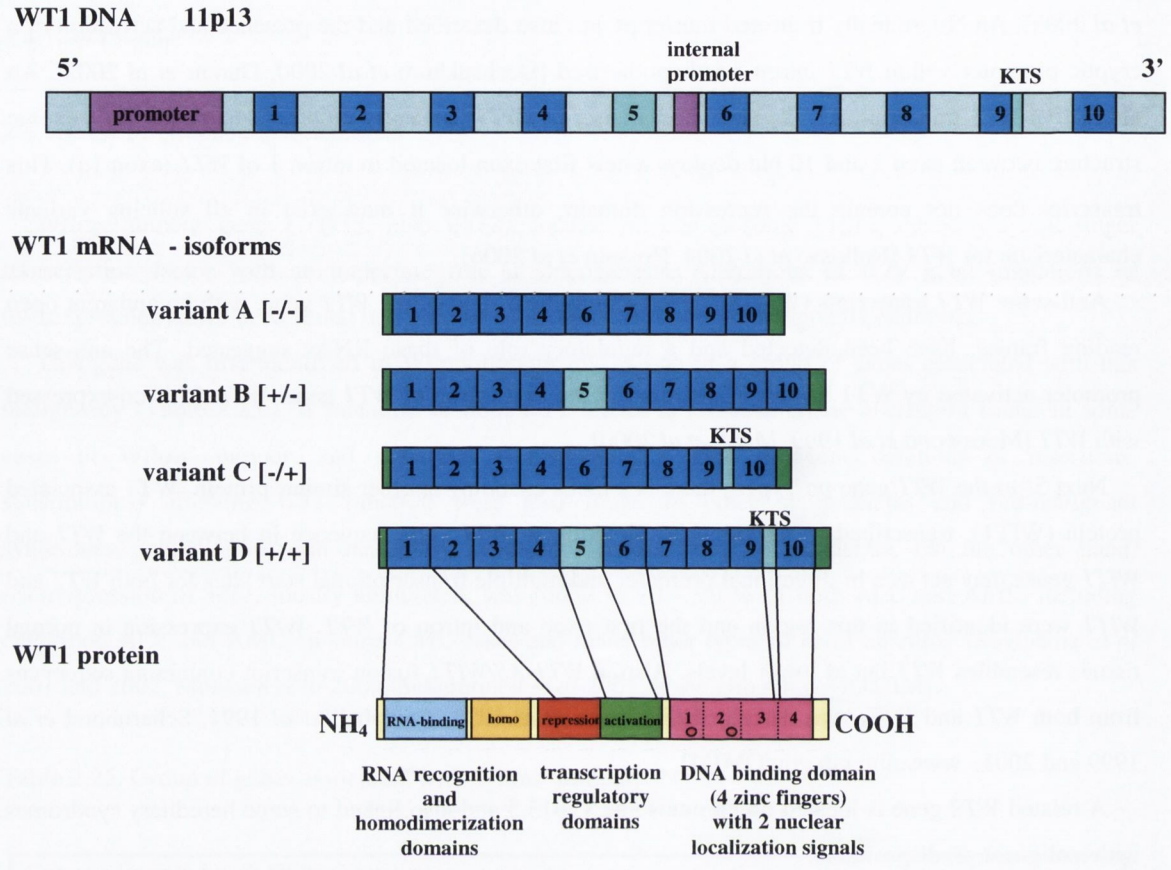


Figure 2.18. WT1 gene and protein.

WT1 proteins appear to perform two main functions: they regulate the transcription of a variety of target genes and are involved in post-transcriptional processing of mRNA. WT1 binds to EGR-1 consensus sequence, a regulatory DNA sequence found in promoters of growth factor genes. EGR-1 is a transcription factor, and WT1 zinc finger DNA binding domain exhibits a considerable homology to that of EGR-1. The regulatory properties of WT1 are not quite well understood. It can act as both a transcriptional activator or repressor of the target genes depending on the cellular and chromosomal context; the actual function may be determined by the WT1 isoform, interactions with other WT1 isoforms, with WT1-interacting protein Par4 and other transcription regulators, including p53, p73, p63, Pax2, NFκB, WTAP (a newly identified WT1-associating protein) or BASP1 (brain acid soluable protein 1) [Little *et al* 2000, Carpenter *et al* 2004, Menke *et al* 2002, Idelman *et al* 2003, Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Wagner *et al* 2003, Scharnhorst *et al* 2001, Karakas *et al* 2002]. Post-translational modifications of WT1, most importantly phosphorylation by protein kinase A and C (PKA and PKC), may also alter its DNA-binding or transcription-regulating properties. The phosphorylation of WT1 has been shown to inhibit WT1 DNA-binding abilities but not the RNA binding. WT1 phosporylation can also change WT1 protein cellular distribution leading to its cytoplasmic retention [Ye

et al 1996]. The function of WT1 as post-transcriptional regulator is suggested by the observations that WT1 can directly bind RNA, interact with constitutive splicing proteins (U2AF65), be incorporated into spliceosomes, is present in nuclear and cytoplasmic ribonucleoprotein particles (RNPs) and can undergo nucleo-cytoplasmic shuttling which is directed by a region of the WT1 containing nuclear export sequences (NES) [Caricasole *et al* 1996, Ladomery *et al* 1999, Vajjhala *et al* 2003, Niksic *et al* 2004]. However, the functional involvement of WT1 in mRNA splicing remains to be elucidated in more detail.

KTS- WT1 isoforms are supposed to be mainly involved in the regulation of transcription. They bind with high affinity to the defined DNA sequence (EGR-1 consensus sequence), can also bind RNA, and display diffuse localisation throughout the nucleus. KTS+ WT1 isoforms are hypothesised to be predominantly involved in RNA processing, probably indirectly as RNA metabolism cofactors. They bind RNA, have reduced DNA binding affinity but can bind a broader range of GC-rich DNA elements, and exhibit a speckled pattern of expression within the nucleus [Englert *et al* 1995, Moorwood *et al* 1999, Sugiyama *et al* 2002, Menssen *et al* 2002, Wagner *et al* 2003, Scharnhorst *et al* 2001]. In haematopoietic system, KTS- WT1 isoforms can arrest normal haematopoietic progenitor cells as well as leukaemic cells in G1 phase and reduce the number of cells in S phase. They also induce differentiation of these cells, which is further enhanced by the presence of KTS+ WT1 isoforms, while inducing quiescence in a primitive subset of haematopoietic precursors [Ellisen *et al* 2001]. Exon 5 (17AA)+ isoforms were found to be responsible for the anti-apoptotic function of WT1, acting in the intrinsic apoptosis pathway [Ito *et al* 2006].

WT1 regulates expression of genes involved in proliferation, differentiation, cell cycle regulation and apoptosis. It controls transcription of growth factor genes, such as *PDGFA* (platelet-derived growth factor), *IGF-2* (insulin-like growth factor), *TGFB* (transforming growth factor), *CSF-1* (colony-stimulating factor) and *EPO* (erythropoietin); growth factor receptor genes, such as *IGF-1-R* (*IGF-1* receptor), *EGFR* (epidermal growth factor receptor); other receptors, such as *RARA* (retinoic acid receptor) and vitamin D receptor; transcription factors like *C-MYB*, *C-MYC* and *N-MYC*, *EGR-1*, *PAX2*, *DAX-1*, *WT1* itself; and other genes like *BCL-2* family, *p21*, cyclin E, *RpAp46* (retinoblastoma suppressor-associated protein), *MDR-1*, *HSP70*, E-cadherin, syndecan 1, amphiregulin and others [Zhang *et al* 1997, Loeb *et al* 2002, Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Scharnhorst *et al* 2001, Karakas *et al* 2002, Cilloni *et al* 2004, Keilholz *et al* 2005, Dame *et al* 2006]. In general, WT1 mostly activates transcription of *EPO*, *RbAp46*, *DAX-1* and antiapoptotic *BCL-2* members and represses transcription of most of the other genes.

The expression of *WT1* gene is controlled by autoregulatory feedback and different regulators. A number of regulators - homeobox genes (PAX2 and 8), NFκB, c-MYB, GATA-1, IGF-1, leukaemia inhibitory factor (LIF), p300 histone acetyltransferase and other factors have been found to modify *WT1* expression [Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Scharnhorst *et al* 2001, Siehl *et al* 2003, Shao *et al* 2005]. *WT1* expression may be also stimulated by hypoxia and HIF-1 (hypoxia-inducible factor-1) and a *HRE* (hypoxia-responsive element) binding HIF-1 has been identified in the *WT1* gene sequence [Wagner *et al* 2003]. Epigenetic control or modification of *WT1* expression was also described. Loss of heterozygosity (LOH) was found in a certain proportion of Wilms' tumours and may be combined with hypermethylation of the other allele as an epigenetic mechanism leading to *WT1* silencing,

which supports the two-hit model of carcinogenesis [Satoh *et al* 2003]. Genomic imprinting (preferential expression of one parental allele due to epigenetic DNA modification - promotor methylation) with a selective expression of the paternal allele has been described in *AWT1* and *WT1-AS*. Relaxation of this imprinting and differential allele expression may occur in malignancies. Previous findings of paternal or biallelic *WT1* expression in normal lymphocytes and fibroblasts and maternal or biallelic *WT1* expression in placenta and foetal brain suggest that the allele-specific regulatory system of *WT1* expression is unique and may be controlled by putative tissue- and individual-specific modifiers [Malik *et al* 2000, www.nlm.nih.gov/OMIM]. CpG promotor or enhancer (hyper)methylation as a mechanism of *WT1* and *WT1-AS* inactivation has also been studied [Kaneuchi *et al* 2005, Dalloso *et al* 2004, Satoh *et al* 2003, Mares *et al* 2001, Loeb *et al* 2001 and 2002]. While some authors found a high correlation between promoter methylation and decrease in *WT1* expression [Kaneuchi *et al* 2005, Satoh *et al* 2003], others could not find any such relation suggesting a different regulation mechanism of *WT1* expression may be employed [Mares *et al* 2001, Loeb *et al* 2001, Wang *et al* 2003].

WT1 is normally expressed only in some tissues, mostly in genitourinary system. Expression of *WT1* was also found in very small subset of normal haematopoietic progenitors under circumstances that are not completely understood and at levels that may be comparable to those in leukaemic cells. This physiologic *WT1* expression is transient and biphasic, limited to very few early uncommitted precursors and then committed progenitors in their late stages of differentiation. The effect of WT1 appears to be stage-specific: WT1 enhances quiescence in more primitive stem cells (the cell cycle arrest is partially mediated by WT1 induction of p21) and induces differentiation in lineage-committed progenitors [Ellisen *et al* 2001, Hosen *et al* 2002]. *WT1* overexpression is present in normal proliferating haematopoietic precursors and may be considered as a surrogate marker of proliferation [Olszewski *et al* 2005]. Proliferation and differentiation of myeloid and erythroid cells is affected by different molecular mechanisms (the zinc finger region of WT1 was shown to be necessary for inhibition of erythroid cell proliferation while it was not involved in inhibition of proliferation and stimulation of differentiation of myeloid cells) [Keilholz *et al* 2005, Svensson *et al* 2005].

2.4.1.2. Role of WT1 in acute leukaemia

Based on its function and other findings, like intragenic deletions or mutations in Wilms' tumours, germ-line mutations in leukaemic predisposition syndromes, and WT1 mediated growth suppression of Wilms' tumour cells expressing a WT1 splicing variant, *WT1* was considered a tumour suppressor gene, although it differs from other tumour suppressor genes, like *RB* or *p53*, by its tissue-restricted expression. Later, it has been proposed that *WT1* gene may have two functional aspects, that of a tumour suppressor gene and an oncogene, and in leukaemias and various types of solid tumours it performs the oncogenic rather than the tumour suppressor gene function. It is possible that the opposing WT1 functions may be mediated by different WT1 molecules, and result from their interactions with other regulatory proteins (e.g. interaction and competition with EGR-1 having an opposite regulatory effect on their common target genes). The oncogenic potential of WT1 is supported by several facts. It has been demonstrated that wild-type, non-mutated *WT1* gene is highly overexpressed in leukaemic blast cells and various types of solid

tumour cells, and increased *WT1* expression is found at leukaemia relapse. Also, there has been shown an inverse correlation between *WT1* expression levels and prognosis in acute leukaemia. Growth of leukaemic and solid tumour cells can be inhibited by treatment with *WT1* antisense oligonucleotides or ribozymes. Myeloid progenitors transfected with *WT1* gene with its constitutive expression showed abnormal response to G-CSF (block of differentiation and induction of proliferation). Ectopic expression of WT1 also suppresses differentiation of myeloid cells induced by vitamin D or retinoic acid. On the other hand, WT1 (overexpressed or in normal levels) was also shown to induce growth arrest and differentiation in leukaemic cells. WT1 can both induce and prevent apoptosis in leukaemic cell lines [Sugiyama *et al* 2001, Menssen *et al* 2002, Scharnhorst *et al* 2001, Ellisen *et al* 2001, Wagner *et al* 2003, Rosenfeld *et al* 2003].

The mechanism of WT1 potential oncogenic effect is not quite clear and the role of WT1 in leukaemogenesis needs to be elucidated. It is possible that deregulated continuous *WT1* expression with discrepancy in the proportion of functionally differing WT1 isoforms may cause the expansion of WT1-expressing normal haematopoietic progenitors and thus contribute to the development of acute leukaemia. The function of WT1 may be exerted in cooperation with other leukaemogenic factors (like AML1-ETO). However, the overexpression of *WT1* in malignant cells may also be a secondary event, a consequence of deregulation of other factors [Sugiyama *et al* 2001, Menssen *et al* 2002, Trka *et al* 2002, Inoue *et al* 1997, Scharnhorst *et al* 2001, Nishida *et al* 2006].

In acute leukaemia, *WT1* is overexpressed in most cases and most leukaemic cells of both ALL and AML, and the overexpression of *WT1* is even more frequent in relapsed acute leukaemias. The up-regulation of *WT1* is present in all leukaemia subtypes, though more differentiated AML subtypes may exhibit lower levels of *WT1* expression than less differentiated, and the frequency of *WT1* expression seems to be higher in B-ALL than in T-ALL [Sugiyama *et al* 2001, Menssen *et al* 1995 and 2002, Niegemann *et al* 1999, Inoue *et al* 1997, Gaiger *et al* 1999, Trka *et al* 2002, Karakas *et al* 2002, Cilloni *et al* 2002 and 2004, Chen *et al* 2001b, Ozgen *et al* 2000]. In some studies, high levels of *WT1* expression at diagnosis were found to be an independent prognostic factor associated with unfavourable prognosis in acute leukaemias, mainly in AML [Trka *et al* 2002, Garg *et al* 2003, Bergmann *et al* 1997, Inoue *et al* 1997, Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Karakas *et al* 2002, Weisser *et al* 2005], but other studies failed to prove this [Gaiger *et al* 1999, Schmid *et al* 1997]. Many studies confirmed the prognostic and predictive value of *WT1* when used as a marker for minimal residual disease monitoring during the disease follow-up [Sugiyama *et al* 1998, Kreuzer *et al* 2001, Menssen *et al* 2002, Trka *et al* 2002, Cilloni *et al* 2002 and 2004, Chen *et al* 2001b, Siehl *et al* 2002, Kletzel *et al* 2002, Garg *et al* 2003, Ostergaard *et al* 2004, Weisser *et al* 2005]. *WT1* was found to be a strong predictor of outcome of allogeneic stem cell transplantation for acute leukaemia [Ogawa *et al* 2003], but not for chronic myeloid leukaemia or BCR-ABL positive acute leukaemia [Uzunel *et al* 2004]. *WT1* overexpression has been also detected in cerebrospinal fluid of patients with B-ALL where it has shown strong association with disease relapse [Ramirez *et al* 2003].

All four main *WT1* variants were found in AML, with significantly higher expression levels of the EX5+ variants [Siehl *et al* 2004]. Also, the *sWT1* transcript, but not the full-length transcript, was found to be overexpressed in leukaemia samples investigated [Hossain *et al* 2006].

The majority of acute leukaemias express normal, wild-type *WT1* gene. In addition to that, point mutations and small deletions or insertions may occur and have been found in about 10 – 20 % of acute leukaemias, predominantly biphenotypic and AML, rarely in ALL. These mutations may affect the zinc finger domains and though they may change the DNA recognition and binding abilities, their biologic significance remains controversial. In AML, *WT1* mutations were shown to be associated with unfavourable prognosis [Drummond *et al* 1994, King-Underwood *et al* 1998, Sugiyama *et al* 2001, Menssen *et al* 2002].

The WT1 protein possesses immunogenic properties and was thus identified as a novel tumour-associated antigen, capable of inducing the anti-tumour immune reaction in *in vitro* and *in vivo* models. This reaction is initiated by helper T lymphocytes stimulated by WT1 peptides and occurs as both cellular and humoral response. Cytotoxic T lymphocytes responding to WT1 protein and anti-WT1-specific antibodies have been also detected in a proportion of patients with acute leukaemia. These findings suggest that WT1 antigen could play an important role in immunotherapy of leukaemia and anti-leukaemic vaccine development [Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Scheibenbogen *et al* 2002, Elisseeva *et al* 2002, Oka *et al* 2002, Bellantuono *et al* 2002, Azuma *et al* 2002, Gao *et al* 2000, Gaiger *et al* 2000, Rosenfeld *et al* 2003]. *In vivo*, the cytotoxic T lymphocyte-mediated specific lysis of leukaemic cells overexpressing WT1 after previous immunisation has been demonstrated in a mouse model [Greiner *et al* 2004]. The clinical trials with WT1 vaccines have already been initiated. An intradermal vaccine containing MHC class I (HLA-A) restricted WT1 peptide (9-mer) in adjuvant was able to increase the number of WT1-specific cytotoxic T lymphocytes, followed by rapid reduction in leukaemic blasts in patients with leukaemia [Oka *et al* 2003 and 2004]. As WT1 is an intracellular protein with predominantly intranuclear localisation, the studies suggest that WT1 protein may be degraded and presented on the target cell surface as peptides in the cleft of MHC molecules to be recognised by specific cytotoxic T lymphocytes [Gaiger *et al* 2000, Rosenfeld *et al* 2003]. MHC class II (HLA-DP5) - restricted Th1 lymphocytes and MHC class I (HLA-A24) - restricted Th1 and Tc1 lymphocytes possessing direct cytotoxic activity against WT1-expressing leukaemic cells were also established [Guo *et al* 2005, Tsuji *et al* 2005].

WT1 might be also a suitable target for new therapeutic strategies using siRNA (small interfering RNA) techniques. In cell lines, *WT1* siRNA induced apoptosis and inhibited proliferation and this effect was further potentiated by *BCR-ABL* siRNA. In normal CD34+ cells, the proliferation was only slightly inhibited and no induction of apoptosis was found [Elmaagacli *et al* 2005].

2.4.2. Detection of WT1 overexpression

The presence and level of *WT1* expression may be detected from RNA extracted from bone marrow, peripheral blood, stem cell harvest, eventually other samples (cerebrospinal fluid) by RT-PCR. After the RT step, PCR (first round, eventually nested) enables qualitative or semi-quantitative evaluation of *WT1* expression [Menssen *et al* 1995, Gaiger *et al* 1999, Cilloni *et al* 2002], while RQ-PCR or competitive PCR give precise quantification of *WT1* expression [Inoue *et al* 1997, Kreuzer *et al* 2001, Cilloni *et al* 2002, Trka *et al* 2002, Ogawa *et al* 2003, Tamaki *et al* 2003, Garg *et al* 2003, Ostergaard *et al* 2004].

PCR reactions for detection of different *WT1* isoforms have been also tested [Dumur *et al* 2002, Siehl *et al* 2004].

The main problem of PCR detection of *WT1* overexpression in acute leukaemia is the lack of standardisation of the reactions used, every author employing a PCR assay of his/her own design with variable specificity and sensitivity, using different, mostly non-standard reaction conditions and different expression of the results. This fact precludes better comparison between the published studies and may be one of the main reasons for the discrepancies in findings between different studies.

The overexpressed WT1 protein may also be detected in nuclear and subnuclear location of leukaemic cells using specific monoclonal antibodies [Menssen *et al* 1995 and 1997].

3. Objectives

This PhD project is a part of a larger study on detection of minimal residual disease in childhood acute leukaemia that has been initiated in 2001 as a cooperative study between Department of Haematology, Institute of Molecular Medicine, Trinity College and St. James' Hospital, Dublin, and Department of Haematology, Our Lady's Hospital for Sick Children, Crumlin, Dublin.

The main objectives of this project are following:

1) Review all available information involving the latest findings in molecular biology of childhood acute leukaemia, prognostic factors in childhood acute leukaemia, particularly minimal residual disease (MRD) and Wilms' tumour gene 1 (*WT1*) overexpression, methods of MRD and *WT1* detection, significance of MRD and *WT1* in childhood acute leukaemia based on both theoretical considerations and results of clinical trials, and investigate other potential prognostic factors and MRD markers or assays for MRD detection.
2) Update and optimise the standard methods of MRD detection based on the recent recommendations of leading working groups (BIOMED-1, BIOMED-2 and EAC groups), results of published studies, and available technology; extend the MRD detection to all childhood acute leukaemia types, including AML.
3) Set up, optimise and validate assays for detection and quantification of *WT1* that could be used as a standard for all further studies on *WT1*.
4) Evaluate all the updated standard methods of MRD detection on patients' samples, obtained from a group of patients with childhood acute leukaemia (25 – 30 patients with 6 - 12 months of follow-up).
5) Evaluate *WT1* expression in diagnostic samples from the above patients with childhood acute leukaemia, and compare with control samples and available samples from patients with other haematological malignancies and pre-malignant diseases.
6) In childhood acute leukaemia patients with *WT1* overexpression in diagnostic samples, use *WT1* as a marker for MRD monitoring and evaluate *WT1* expression in all follow-up samples.
7) Correlate the level of *WT1* overexpression at diagnosis with other prognostic factors and with clinical outcome; correlate the different methods of MRD detection together and with clinical data.
8) Make conclusions and recommendations for the future use of *WT1* detection and MRD monitoring in childhood acute leukaemia in routine laboratory practice.

4. Methods

Samples

4.1. Cell lines

Cell lines were used for the very initial FC work (establishment of the method), set up and optimisation of PCR and RQ-PCR of chromosomal aberrations including sensitivity and specificity tests, set up and optimisation of detection of *WT1* overexpression by PCR, RQ-PCR, FC and IFM/IHC (immunofluorescence microscopy, immunohistochemistry), and related sensitivity and specificity tests.

They were further used as positive and negative controls for PCR and RQ-PCR of chromosomal aberrations and as positive controls for PCR and RQ-PCR of *WT1* overexpression.

In total, 14 different human leukaemia cell lines were grown, used for the experiments, and frozen stocks prepared for future needs (Table 4.1.).

Table 4.1. Cell lines used in the experiments.

Cell line	Cell type	Cytogenetic/molecular characteristics	Source
HL-60	AML	none of interest	DSMZ, ACC 3
MOLT-3	T-ALL	none of interest	DSMZ, ACC 84
SD-1	B-ALL	t(9;22) BCR-ABL p190 (e1-a2, e1-a3)	DSMZ, ACC 366
K-562	CML	t(9;22) BCR-ABL p210 (b3-a2)	DSMZ, ACC 10
BV-173	B-ALL	t(9;22) BCR-ABL p210 (b2-a2)	DSMZ, ACC 20
KY-01	CML	t(9;22) BCR-ABL p210 (b2-a2)	Prof. Melo J., Hammersmith Hospital, London, UK
REH	B-ALL	t(12;21) TEL-AML1	DSMZ, ACC 22
MV4-11	AML	t(4;11) MLL-AF4 (e9-e5)	DSMZ, ACC 102
697	B-ALL	t(1;19) E2A-PBX1	DSMZ, ACC 42
CCRF-CEM	T-ALL	microdel (1p) SIL-TAL1 (1.a1b-3.4, 1a-3.4)	DSMZ, ACC 240
MOLT-16	T-ALL	microdel (1p) SIL-TAL1	DSMZ, ACC 29
NB-4	AML	t(15;17) PML-RARA (bcr 1)	DSMZ, ACC 207
KASUMI-1	AML	t(8;21) AML1-ETO	DSMZ, ACC 220
ME-1	AML	inv(16) CBFB-MYH11 (type A)	Dr. B. A. Van der Reijden, University Medical Centre, Nijmegen, NL

(DSMZ – German Collection of Microorganisms and Cell Cultures)

4.2. Patients

4.2.1. Group of patients for MRD study

After informed consent (ethical committee approval obtained before the beginning of the study), 25 patients with childhood acute leukaemia aged over 1 year, diagnosed in the Our Lady's Hospital for Sick Children (OLHSC) in Crumlin, Dublin, from June 2003 to March 2004 were enrolled on prospective sequential basis (infants less than 1 year of age were not entered into the study). They included 23 patients with newly diagnosed childhood acute leukaemia (19 B-ALL, 3 T-ALL and 1 AML) and 2 patients with a relapse of childhood acute leukaemia (1 B-ALL and 1 AML). The male/female ratio was close to 1 (12 males, 13 females, 1 : 1.1), the median age was 5 years, ranging from 1 to 16 years. Main risk factors involving the age ≥ 10 years, WBC ≥ 50 x 10^9 / l or hypodiploidy were present in 4 (17 %), 7 (30 %) and 1 (4 %) pts, respectively (Table 4.2.). The patients with ALL were stratified into risk groups and treated within the UKALL 97/99 MRC trial (Table 4.3.). At diagnosis, 12 patients (52 %) were stratified into the standard risk group A, 10 patients (43 %) into the intermediate risk group B and 1 patient (4 %) into the high risk group C. After assessment of the initial bone marrow response, 2 patients from the group B were switched into the group C. The patients with AML were treated within the AML-15 MRC trial. One patient with AML underwent bone marrow transplantation (BMT) after induction therapy. The median follow-up was 12 months (range 1 - 17 months). During this time no patient relapsed, but 5 patients (20 %) died, 2 of them (8 %) due to treatment complications (sepsis) and 3 pts (12 %) of leukaemia progression (Table 4.2.).

From each patient, a bone marrow sample from the time of diagnosis and 1 – 8 follow-up samples from different time points up to 1 year after the diagnosis (day 8, 15 and 29 - D8, D15 and D29; week 7 to 61 -w7 to w61) were obtained. Only in 1 patient, the diagnostic sample was not available so D8 sample was used instead of the diagnostic one for the identification of MRD markers. This patient was not included into the assessment of *WT1* expression at diagnosis. In 3 patients, a peripheral blood sample from the time of diagnosis was also available. In total, 24 diagnostic and 138 follow-up bone marrow samples, and 3 peripheral blood diagnostic samples were collected and investigated.

4.2.2. Group of patients for WT1 study

All the patients above were also evaluated in the WT1 study. In addition to them, further patients were included: Two more patients with newly diagnosed childhood acute leukaemia (1 B-ALL, 1 T-ALL) were enrolled in April and May 2004 for evaluation of *WT1* expression at diagnosis only. In these patients, only diagnostic bone marrow samples were obtained (Table 4.2.). *WT1* expression at diagnosis was analysed in stored RNA samples extracted from bone marrow of childhood ALL patients from OLHSC who were entered into the previous part of the larger MRD project since January 2001. Thirty-seven of these RNA samples (30 B-ALL, 7 T-ALL) were available and investigated. Thus, in the initial phase, *WT1* expression at diagnosis was evaluated in 63 childhood acute leukaemia patients, 50 B-ALL (including 1 relapse), 11 T-ALL and 2 AML.

Table 4.2. Overview of the data of patients enrolled into the study - cont.

UPN	Sex	Age [y]	Dg	Risk group	Dg sample	Follow up samples									Follow up [m]	Risk factors*)	Relapse	Death	Other
1	F	3	B-ALL	A	BM	D8	D15	D29	w7	w16	w24	w33	w43		14				
2	M	1	B-ALL	A	BM	D8		D29	w7	w19					12				
3	F	11	B-ALL	B-C	BM	D8	D15	D29	w12	w20	w30				**9**	A		+, sepsis	
4	F	4	B-ALL	B	BM	D8	D15	D29		w20	w31	w38	w49		13	W			
5	F	5	B-ALL	B	BM	D8		D29	w12	w21	w26	w35	w45		13	W			
6	M	3	B-ALL	A	BM (+F) + PB	D8	D15	D29	w7	w16	w24	w32	w41		13				
7	F	4	B-ALL	C	BM	D8	D15	D29	w12	w21	w29	w37			10	H			
8	M	9	B-ALL	A	BM-F	D8	D15	D29		w16	w24	w32	w43		12				
9	F	14	B-ALL	B	BM + PB	D8	D15	D29							**3**	A, W		+, sepsis	
10	M	5	B-ALL	A	BM	D8	D15	D29	w8	w16	w27	w34			10				
11	F	16	B-ALL	B	BM	D8	D15	D29	w11	w18	w37				10	A			
12	F	6	B-ALL	B-C	BM	D8	D15	D29			w26	w36	w44		14	W			
13	M	4	B-ALL	A	BM	D8	D15	D29	w7	w16	w25	w30	w40		13				
14	F	5	B-ALL	A	BM	D8	D15	D29							9				
15	M	8	B-ALL	A	BM	D8	D15	D29	w8	w17	w32	w39	w50		12				
16	F	4	B-ALL	A	BM-F	D8		D29	w7		w26	w38	w47	w61	15				
17	M	4	B-ALL	A	BM (+F)	D8	D15	D29	w7	w16	w26	w34	w43		17				
18	M	12	B-ALL	B	BM + PB	D8	D15	D29	w13	w21	w30	w39	w53		16	A			
19	F	5	B-ALL	A	no	D8	D15	D29							8				
20	M	7	B-ALL-R1	(A)	BM	D8									**1**			+, relapse	
21	M	8	T-ALL	B	BM-F	D8	D15	D29							8	W			
22	F	3	T-ALL	B	BM-F	D8	D15	D29	w10	w18	w27	w39	w52		16	W			
23	F	1	T-ALL	B	BM	D8	D15								**1**	W		+, ALL	
24	M	1	AML	-	BM	D8			w7						16				BMT
25	M	4	AML-R1 (post BMT)	-	BM				w11						**3**			+, relapse	

Table 4.2. Overview of the data of patients enrolled into the study.

26	M	7	B-ALL	B	BM	no	7				
27	F	5	T-ALL	B	BM	no	6				

UPN – unique patient number, ALL, AML-R1 - 1st relapse; BM - bone marrow, PB - peripheral blood, BM-F - only frozen BM sample available from the time of diagnosis, BM (+F) - BM and frozen BM sample available from the time of diagnosis; *) risk factors: A - age ≥ 10 years, W - WBC ≥ 50 x 10^9 / l, H - hypodiploidy

Table 4.3. UKALL 97/99 MRC trial - risk stratification.

UKALL 97/99 MRC trial - risk stratification	
standard risk (regimen A)	• age > 1 and < 10 and • Dg WBC < 50 x 10^9/l and • no hypodiploidy (≤ 44 chromosomes) no BCR-ABL gene rearrangements no MLL gene rearrangements (age 1 –2) and • < 25 % of blasts in BM on D15
intermediate risk (regimen B)	• age ≥ 10 or • Dg WBC ≥ 50 x 10^9/l and • no hypodiploidy (≤ 44 chromosomes) no BCR-ABL gene rearrangements no MLL gene rearrangements (age 1 – 2) • < 25 % of blasts in BM on D8
high risk (regimen C)	• hypodiploidy (≤ 44 chromosomes) BCR-ABL gene rearrangements MLL gene rearrangements (age 1 – 2)
+ evaluation of initial response:	
group A	< 25 % of blasts in BM d15
group B	< 25 % of blasts in BM d8
group C	if not above, switch to C

In the next phase of WT1 study, *WT1* expression was evaluated in a representative group of newly diagnosed childhood ALL patients from 2 centres: 60 patients from OLHSC Crumlin, Dublin (described previously), and 65 patients from University Hospital in Prague - Motol, Czech Republic. Diagnostic bone marrow samples of the enrolled Czech patients were collected after the local ethical committee approval on a consecutive basis from February 2001 to September 2002 for molecular studies and in the vast majority of cases there was enough RNA or cDNA left for the *WT1* detection. The characteristics of the Czech and Irish group were similar (Table 4.4.). The Czech patients were treated according to ALL-BFM 95, POG9407 and Interfant protocols.

In the whole cohort the male/female ratio was 1.3 : 1, the median age was 5 years (range 0 - 16 years), the diagnosis included 106 B-ALL (including 1 hybrid ALL) and 19 T-ALL cases. Among them, 27 patients had chromosomal translocations confirmed by cytogenetics and/or PCR: 22 *TEL-AML1* (*ETV6-RUNX1*), 3 *MLL-AF4* and 2 *BCR-ABL* (no patient from the Irish group expressed *E2A-PBX1* translocation, Czech patients were not screened for this aberration). Main risk factors defined as age ≤ 1 or ≥ 10 years and WBC ≥ 50 x 10^9 / l at diagnosis were present in 3, 35 and 30 patients, respectively. The

median follow-up is 32 months (range 1 - 47); during this period 13 of the 120 patients relapsed, 1 showed no response with continual disease progression since diagnosis, and 11 patients died (Table 4.4.).

Because of the small number of patients with *MLL-AF4* and *BCR-ABL* translocations enrolled sequentially, further patients with these translocations (1 *MLL-AF4* and 9 *BCR-ABL*, all B-ALL) were selected retrospectively from Czech patients who had stored bone marrow RNA available. These cases were included only in analysis of *WT1* expression in different chromosomal translocations and in correlation of *WT1* and CD34 expression.

Table 4.4. Characteristics of the group of patients included into WT1 study.

Characteristics	IRL	CZ	total
Patients:			
total no.	60	65	125
M/F	26 / 34 (1 : 1.3)	44 / 21 (2.1 : 1)	70 / 55 (1.3 : 1)
age	5 (1 – 16)	5 (0 – 16)	5 (0 – 16)
Dg:			
B-ALL	49	56	105
T-ALL	11	8	19
hybrid	0	1	1
Chromosomal aberrations:			
TEL-AML1	7	15	22
MLL-AF4	0	3	3
BCR-ABL	0	2	2
Risk factors:			
age ≥ 10	18	17	35
age ≤ 1	0	3	3
WBC ≥ 50	16	14	30
Follow-up:			
Length	24 (1 – 46)	36 (1 – 47)	32 (1 – 47)
Relapse/progressive	5	9	14
- BM±oth	3	7	10
- CNS	1	1	2
- testes	0	1	1
- progressive	1	0	1
Died	6	5	11
- of relapse/progress	3	3	6
- other	3	2	5

4.3. Donors

Donors' samples were used as negative controls for all FC, PCR and RQ-PCR experiments, as well as for IFM/IHC tests. They were also used for specificity and sensitivity tests. For specificity tests, they were particularly important for the assessment of the background expression levels of the investigated targets like leukaemia-associated immunophenotypes (LAPs), *IG/TCR* clonality and *WT1* expression. For sensitivity tests, patient's cells, DNA or RNA were diluted into donor's cells, DNA or RNA, which should best reflect the sensitivity in real conditions.

Following donors' samples were used:

1) normal peripheral blood
   - obtained from healthy adult donors from Transfusion Services and from colleagues in the lab, and from adult patients from St. James's Hospital with non-haematological diseases
2) normal bone marrow
   - obtained from healthy adult bone marrow donors from St. James's Hospital
3) normal peripheral stem cells
   - obtained from healthy adult peripheral stem cell donors from St. James's Hospital
4) bone marrow of childhood patients with other haematological malignancies and non-malignant diseases
   - obtained from childhood patients from OLHSC Crumlin
5) regenerating bone marrow of patients with childhood acute leukaemia
   - obtained at various time-points from the follow-up samples of the patients enrolled to this study, providing there was enough material and the bone marrow was confirmed to be MRD negative (by other method) or there was no expression of the target of interest (e.g. bone marrow samples of B-ALL patients used as controls for evaluation of T-ALL-related LAPs expression and vice versa, bone marrow samples of different patients used as controls for *IG/TCR* clonality tests for other patients, bone marrow samples of patients without chromosomal aberrations used as controls for detection of different chromosomal aberrations, MRD negative bone marrow samples used as controls for assessment of abnormal levels of *WT1* expression, etc.)

Techniques:

4.4. Cell culture

4.4.1. Defrosting cells and starting initial cultures

All cell lines were obtained as frozen cultures. The cells were very slowly thawed on ice, immediately transferred into a large volume of cold RPMI medium and washed. The cell pellet was then resuspended in pre-warmed 20 % complete RPMI medium and transferred into flasks for the initial culture. Cells were grown in the incubator at 37 °C and 5 % CO_2 for 2 - 4 days (eventually more depending on the cell type). When the culture reached the appropriate cellular density, the first passaging was performed (Protocol 1).

4.4.2. Passaging cells in cultures

With the exception of CCRF-CEM cell line, all the other cell lines were growing in suspension. The contents of a flask with the cell suspension was transferred into a sterile tube, centrifuged, supernatant discarded, the cell pellet was resuspended in an appropriate amount of pre-warmed 10 % or 20 % complete RPMI medium (depending on the cell type), and a part of this suspension was then transferred into new flask(s) pre-filled with warm 10 % or 20 % complete RPMI medium. Cells were grown in the incubator at 37 °C and 5 % CO_2 for 2 - 4 days (depending on the cell type), then the next passaging was performed (Protocol 2A).

CCRF-CEM cell line grows in suspension and as adherent cells forming a monolayer. The cells growing in suspension were handled as described previously. In addition, the adherent cells were removed by trypsinising and scraping. After removing the medium with cell suspension, trypsin was added into the flask and incubated for 5 min at 37 °C. Following addition of a small volume of HBSS medium (to terminate the enzyme activity), the flask surface was scraped with a sterile scraper and washed with more HBSS. The whole contents of the flask was then transferred into a sterile tube and processed along with the other cell suspension (Protocol 2B).

As a part of the laboratory policy, mycoplasma testing of all cell cultures was carried out every three months. All cultures always were found to be negative.

4.4.3. Freezing cells

After several passages, a frozen stock of every cell line was prepared. The cell suspension was centrifuged and the cell pellet resuspended in an appropriate amount of pre-warmed 10 % or 20 % complete RPMI medium. The cells were counted, the concentration adjusted to 5 - 10 x 10^6 cells / ml, and placed on ice. Freezing medium 2X, containing RPMI medium, FBS and DMSO, was prepared and cooled on ice. The same volume of the ice-cold 2X freezing medium was slowly, drop-wise with permanent mixing, added to the cell suspension. This suspension was then aliquoted into sterile cryo-vials

on ice, put in a polystyrene box (allowing them to cool at - 1 °C per min) and frozen at - 80 °C. The next day or as soon as possible, the frozen cells were transferred and stored in liquid nitrogen (- 196 °C) (Protocol 3).

4.4.4. Using cells for experiments

For all further experiments, the cells were centrifuged, the cell pellet resuspended in the relevant solution, the cells were counted (Protocol 4) and their concentration adjusted as required. They were then used according to the further protocols.

4.5. Sample collection

Bone marrow, peripheral blood or peripheral stem cell samples (approx. 1 ml of bone marrow or peripheral stem cell concentrate, and 2 - 10 ml of peripheral blood) were collected into tubes with an anticoagulant solution (ACD, K_2 or K_3EDTA) - for PCR tests, or into tubes containing 10 ml of RPMI medium - for FC analysis.

During the course of the study, several different types of RNA stabilizing solutions became commercially available and would be of interest for RNA preservation during the sample transport. At present, none is designed specifically for RNA stabilization in bone marrow samples. Those available (Qiagen and other companies) are recommended for use with either peripheral blood or other tissue samples, however after validation tests they could be used also with bone marrow samples (Qiagen technical services manager, personal communication). For further studies, especially in case of cooperative studies between different hospitals, the issue of RNA stabilization may require attention and further evaluation.

After collection, samples were transported into the lab as fast as possible, at room temperature. The initial processing of the samples was done immediately, within 24 hours at most, ideally and in most of the cases within 4 hours after their collection. This included mononuclear cell separation, preparation of cell lysates for RNA and DNA extraction and their storage at - 80 °C, labelling cells for FC and their acquisition on the flow cytometer.

4.6. Mononuclear cell separation

Mononuclear cells from bone marrow, peripheral blood or peripheral stem cell concentrate were separated on a density gradient medium (Lymphoprep). Lymphoprep is a solution containing sodium metrizoate and polysaccharide, which aggregates the erythrocytes, thereby increasing their sedimentation rate. The sedimentation of mononuclear cells is only slightly affected and, after centrifugation, they form a layer above the erythrocytes, well separated for collection.

Bone marrow, peripheral blood or stem cells were diluted in D-PBS (except bone marrow previously diluted in RPMI) and carefully layered over Lymphoprep. After centrifugation, the mononuclear-rich interphase was collected and the cells washed in D-PBS. The cells were then resuspended in the appropriate solution, counted and their concentration adjusted as required. This suspension of cells was used for further experiments (Protocol 5).

4.7. RNA extraction

RNA was isolated from the cells using Qiagen RNeasy Mini Kit. The cells are first lysed with GITC (guanidine isothiocyanate)-containing lysing buffer, which immediately inactivates RNases. The sample is homogenised to shear genomic DNA and reduce viscosity of the lysate, ethanol is added to provide appropriate binding conditions and the sample is then applied to a column containing a silica-gel-based membrane. In the presence of the high-salt buffer system (in the lysate) this membrane selectively binds RNA longer than 200 bases. Contaminants are removed by several washing steps and remaining DNA is degraded by incubation with DNase. High-quality RNA is then eluted in sterile distilled water.

RNA extraction was performed according to the RNeasy Mini Handbook (Qiagen, June 2001). Lysing solution was added to the cell suspension and the cell lysate was stored at - 80 °C. Later, the cell lysate was thawed and RNA extracted following the recommended protocol. The eluted RNA was checked for the purity and quality and stored at - 80 °C (Protocol 6).

4.8. DNA extraction

DNA was isolated from the cells using Qiagen QIAamp DNA Blood Mini Kit. The cells are first lysed with a lysing buffer with an adjusted concentration of salts and pH. The lysate is incubated with protease or proteinase K to release the DNA from the cells and nuclear proteins. The sample is then applied to a column containing a silica-gel-based membrane, which (under the optimal conditions induced by the lysing buffer) selectively binds DNA. Remaining contaminants are removed by several washing steps. High-quality DNA is then eluted in a buffer.

DNA extraction was performed according to the QIAamp DNA Blood Mini Handbook (Qiagen, February 2003). D-PBS and lysing solution were added to the cell suspension and the cell lysate was stored at - 80 °C. Later, the cell lysate was thawed and DNA extracted following the recommended protocol. The eluted DNA was stored at - 80 °C (Protocol 7).

4.9. Detection of leukaemia-associated phenotypes by flow cytometry

Four-colour flow cytometry (FC) with separate antibody panels for B-ALL, T-ALL and AML has been used for detection of minimal residual disease (MRD) by detection of leukaemia-associated phenotypes (LAPs). The principle of flow cytometric detection of MRD by immunophenotyping and detection of LAPs is described in chapter 2.3.2.1. As there are currently no standardised protocols for detection of MRD in acute leukaemia by flow cytometry, the method was set up based on the latest knowledge in this area, recommendations for immunophenotyping of acute leukaemia, and methodology and results of recently published studies [Bain *et al* 2002, Lucio *et al* 1999 and 2001, Owens *et al* 2000, Campana *et al* 1999, 2000, 2001, 2002 and 2003, San Miguel *et al* 2001 and 2002, Vial *et al* 2001, Ito *et al* 2001, Kaleem *et al* 2003, Porwit-MacDonald *et al* 2000, Khalidi *et al* 1999, Legrand *et al* 2000, Bene *et al* 2001, Scolnik *et al* 2002, Veltroni *et al* 2003, Orfao *et al* 1999, Rizzatti *et al* 2002, Wuchter *et al* 2000, van Lochem *et al* 2000, van Wering *et al* 2000, Oelschlaegel *et al* 2000, Baer *et al* 2001, Coustan-Smith *et al* 2000, 2002b and 2003, Dworzak *et al* 2002, Venditti *et al* 2000, Kern *et al* 2003, Reinhardt *et al* 2002, Sievers *et al* 2003].

4.9.1. Antibody panels

Separate antibody panels were designed for B-ALL, T-ALL and AML and LAPs defined for each of the antibody 4-combination. They include the most common as well as rarer LAPs, both quantitative and qualitative. Unlabelled cells and isotype controls were included as intra-assay controls (Table 4.5.).

For detection of all antigens, mouse anti-human monoclonal antibodies were used. To achieve their best performance, only antibodies of high quality, preferably of IgG1 isotype (giving the lowest unspecific binding), and in amounts optimised by titration were employed.

The antibodies were conjugated with 4 different fluorochromes: fluorescein (FITC), R-phycoerythrin (PE) and phycoerythrin-cyanine 5 conjugate (PECy5) excited by argon laser (488 nm), and allophycocyanin (APC) excited by helium-neon laser (650 nm). PECy5 instead of peridinin-chlorophyll protein (PerCP) was chosen as the third colour because of stronger fluorescence signal and also a wider range of antibodies available in conjugation with this fluorochrome than with PerCP.

Table 4.5. Four-colour FC antibody panels for detection of MRD in B-ALL, T-ALL and AML; antibodies used for set up of compensation parameters in B-ALL, T-ALL and AML - cont.

B-ALL PANEL

Surface markers:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
1.	Neg. Ctrl.	CD19-APC	IgG1	neat	5 µl	DAKO
2.	Izotype Ctrl.	IgG1-FITC		neat	5µl	DAKO
		IgG1-PE		neat	5µl	DAKO
		IgG1-PECy5		neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
3.	CD38/CD34/CD45/CD19	CD38-FITC	IgG1	neat	5µl	DAKO
	LAP: quantitative	CD34-PE	IgG1	neat	5µl	DAKO
		CD45-PECy5	IgG1	neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
4.	CD22/CD34/CD20/CD19	CD22-FITC	IgG1	neat	5µl	DAKO
	LAP: quantitative	CD34-PE	IgG1	neat	5µl	DAKO
		CD20-PECy5	IgG1	neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
5.	CD21/CD10/CD34/CD19	CD21-FITC	IgG1	neat	5µl	DAKO
	LAP: 21+/10+, 21+/34+,	CD10-PE	IgG1	neat	5µl	DAKO
	quantitative	CD34-PECy5	IgG1	neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
6.	Izotype Ctrl.	IgG2a-FITC		neat	5µl	Serotec
		IgG1-PE		neat	5µl	DAKO
		IgG1-PECy5		neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
7.	CD58/CD10/CD34/CD19	CD58-FITC	IgG2a	neat	5µl	Serotec
	LAP: 58+/10+, 58+/34+	CD10-PE	IgG1	neat	5µl	DAKO
	(quantitative)	CD34-PECy5	IgG1	neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
8.	CD56/CD4//CD19	CD56-FITC	IgG2a	neat	5µl	Serotec
	LAP: 56+/19+, 4+/19+	CD4-PE	IgG1	neat	20µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
9.	Izotype Ctrl.	IgM-FITC		neat	20 µl	BC
		IgG1-PE		neat	5µl	DAKO
		IgG1-PECy5		neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
10.	CD65/CD10/CD66/CD19	CD65-FITC	IgM	neat	20 µl	BC
	LAP: 65+/19+, 66+/19+	CD10-PE	IgG1	neat	5µl	DAKO
		CD66-PECy5	IgG1	neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
11.	CD15/CD33/CD13/CD19	CD15-FITC	IgM	neat	20 µl	BC
	LAP: 15+/19+, 33+/19+,	CD33-PE	IgG1	neat	5µl	DAKO
	13+/19+	CD13-PECy5	IgG1	neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
12.	Izotype Ctrl.	IgG1-FITC		neat	5µl	DAKO
		IgG1-PE		neat	5µl	DAKO
		IgG2aPECy5		neat	5µl	Serotec
		CD19-APC	IgG1	neat	5µl	DAKO
13.	CD2/CD5/CD7/CD19	CD2-FITC	IgG1	neat	5µl	DAKO
	LAP: 2+/19+, 5+/19+,	CD5-PE	IgG1	neat	5µl	DAKO
	7+/19+	CD7-PECy5	IgG2a	½	5µl	Serotec
		CD19-APC	IgG1	neat	5µl	DAKO
14.	Izotype Ctrl.	IgG1-PE		neat	20µl	BC
		CD19-APC	IgG1	neat	5µl	DAKO
15.	m7.1//CD19	m7.1-PE	IgG1	neat	20µl	BC
	LAP: m7.1+/19+	CD19-APC	IgG1	neat	5µl	DAKO

Table 4.5. Four-colour FC antibody panels for detection of MRD in B-ALL, T-ALL and AML; antibodies used for set up of compensation parameters in B-ALL, T-ALL and AML - cont.

B-ALL PANEL

Intracellular markers:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
16.	Neg. Ctrl.	CD19-APC	IgG1	neat	5 µl	DAKO
17.	Izotype Ctrl.	*F(ab)$_2$-FITC		1/7	5µl	DAKO
		IgG1-PE		neat	5µl	Serotec
		IgG1-PECy5		neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO
18.	cIgM/CD10/CD34/CD19	*cIgM-FITC	F(ab)$_2$	1/10	5µl	DAKO
	LAP: cIgM+/34+,	CD10-PE	IgG1	neat	5µl	Serotec
	quantitative	CD34-PECy5	IgG1	neat	5µl	DAKO
		CD19-APC	IgG1	neat	5µl	DAKO

* - intracellular (cytoplasmatic, nuclear) Ag

Compensations:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
C1.	Neg. Ctrl.	no				
C2.	Compenzation – FITC	CD45-FITC	IgG1	neat	5 µl	DAKO
C3.	Compenzation – PE	CD45-PE	IgG1	neat	5 µl	DAKO
C4.	Compenzation – PECy5	CD45-PECy5	IgG1	neat	5 µl	DAKO
C5.	Compenzation – APC	CD45-APC	IgG1	neat	5 µl	DAKO
C6.	Izotype Ctrl. - gating	IgG1-APC		neat	5 µl	DAKO

Table 4.5. Four-colour FC antibody panels for detection of MRD in B-ALL, T-ALL and AML; antibodies used for set up of compensation parameters in B-ALL, T-ALL and AML - cont.

T-ALL

Surface markers:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
1.	Neg. Ctrl.	CD7-PECy5	IgG2a	1/2	5 µl	Serotec
2.	Izotype Ctrl.	IgM-FITC		neat	20µl	BC
		IgG1-PE		neat	5µl	Serotec
		CD7-PECy5	IgG2a	1/2	5µl	Serotec
		IgG1-APC		neat	5µl	DAKO
3.	CD15/CD4/CD7/CD8	CD15-FITC	IgM	neat	20µl	BC
	LAP: 4+/8+, 4-/8-, 15+/7+	CD4-PE	IgG1	neat	5µl	DAKO
		CD7-PECy5	IgG2a	1/2	5µl	Serotec
		CD8-APC	IgG1	neat	5µl	DAKO
4.	Izotype Ctrl.	IgG1-FITC		neat	5µl	DAKO
		IgG1-PE		neat	5µl	Serotec
		CD7-PECy5	IgG2a	1/2	5µl	Serotec
		IgG1-APC		neat	5µl	DAKO
5.	CD21/CD10/CD7/CD5	CD21-FITC	IgG1	neat	5µl	DAKO
	LAP: 21+/7+, 10+/7+,	CD10-PE	IgG1	neat	5µl	Serotec
	quantitative	CD7-PECy5	IgG2a	1/2	5µl	Serotec
		CD5-APC	IgG1	neat	5µl	DAKO
6.	Izotype Ctrl.	IgG2a-FITC		neat	5µl	Serotec
		IgG1-PE		neat	5µl	DAKO
		CD7-PECy5	IgG2a	1/2	5µl	Serotec
		IgG1-APC		neat	5µl	DAKO
7.	CD1a/CD13/CD7/CD3	CD1a-FITC	IgG2a	neat	5µl	Serotec
	LAP: 1a+/3+, 13+/7+, 13+/3+	CD13-PE	IgG1	neat	5µl	DAKO
		CD7-PECy5	IgG2a	1/2	5µl	Serotec
		CD3-APC	IgG1	neat	5µl	DAKO

Intracellular markers:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
8.	Neg. Ctrl.	CD7-PECy5	IgG2a	1/2	5 µl	Serotec
9.	Izotype Ctrl.	*IgG1-FITC		neat	5µl	DAKO
		*IgG1-PE		neat	5µl	DAKO
		CD7-PECy5	IgG2a	1/2	5µl	Serotec
		IgG1-APC		neat	5µl	DAKO
10.	TdT/cCD3/CD7/CD3	*TdT-FITC	IgG1	neat	5µl	DAKO
	LAP: cytTdT+/cyt3+,	*CD3-PE	IgG1	neat	5µl	DAKO
	cytTdT+/3+, 7+/3-, 7-/3+	CD7-PECy5	IgG2a	1/2	5µl	Serotec
		CD3-APC	IgG1	neat	5µl	DAKO
11.	Izotype Ctrl.	IgG1-FITC		neat	5µl	DAKO
		*IgG1-PE		neat	5µl	DAKO
		CD7-PECy5	IgG2a	1/2	5µl	Serotec
		IgG1-APC		neat	5µl	DAKO
12.	CD34/cCD3/CD7/CD3	CD34-FITC	IgG1	neat	5µl	DAKO
	LAP: 34+/cyt3+, 34+/3+,	*CD3-PE	IgG1	neat	5µl	DAKO
	7+/3-,	CD7-PECy5	IgG2a	1/2	5µl	Serotec
	7-/3+	CD3-APC	IgG1	neat	5µl	DAKO

* - intracellular (cytoplasmatic, nuclear) Ag

Table 4.5. Four-colour FC antibody panels for detection of MRD in B-ALL, T-ALL and AML; antibodies used for set up of compensation parameters in B-ALL, T-ALL and AML - cont.

T-ALL PANEL

Compensations:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
C1.	Neg. Ctrl.	no				
C2.	Compenzation – FITC	CD45-FITC	IgG1	neat	5 µl	DAKO
C3.	Compenzation – PE	CD45-PE	IgG1	neat	5 µl	DAKO
C4.	Compenzation – PECy5	CD7-PECy5	IgG2a	1/2	5 µl	Serotec
C5.	Compenzation – APC	CD45-APC	IgG1	neat	5 µl	DAKO
C6.	Izotype Ctrl. - gating	IgG2a-PECy5		neat	5 µl	Serotec

Table 4.5. Four-colour FC antibody panels for detection of MRD in B-ALL, T-ALL and AML; antibodies used for set up of compensation parameters in B-ALL, T-ALL and AML - cont.

AML PANEL

Surface markers:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
1.	Neg. Ctrl.	CD45-PECy5	IgG1	neat	5 µl	DAKO
2.	Izotype Ctrl.	IgG1-FITC		neat	5 µl	DAKO
		IgG1-PE		neat	5 µl	DAKO
		CD45-PECy5	IgG1	neat	5 µl	DAKO
		IgG1-APC		neat	5 µl	DAKO
3.	CD34/CD13/CD45/CD117	CD34-FITC	IgG1	neat	5 µl	DAKO
	LAP: 13+/117+, quantitative	CD13-PE	IgG1	neat	5 µl	DAKO
		CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
4.	CD34/CD11b/CD45/CD117	CD34-FITC	IgG1	neat	5 µl	DAKO
	LAP: 11b+/34+, 11b+/117+	CD11b-PE	IgG1	neat	5 µl	DAKO
		CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
5.	CD13/CD19/CD45/CD117	CD13-FITC	IgG1	neat	5 µl	DAKO
	LAP: 19+/13+, 19+/117+,	CD19-PE	IgG1	neat	5 µl	DAKO
	13+/117+	CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
6.	CD2/CD13/CD45/CD117	CD2-FITC	IgG1	neat	5 µl	DAKO
	LAP: 2+/13+, 2+/117+,	CD13-PE	IgG1	neat	5 µl	DAKO
	13+/117+	CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
7.	CD13/CD4/CD45/CD117	CD13-FITC	IgG1	neat	5 µl	DAKO
	LAP: 4+/13+, 4+/117+,	CD4-PE	IgG1	neat	5 µl	DAKO
	13+/117+	CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
8.	CD13/CD33/CD45/CD117	CD13-FITC	IgG1	neat	5 µl	DAKO
	LAP: 13+/33-, 13-/33+,	CD33-PE	IgG1	neat	5 µl	DAKO
	13+/117+	CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
9.	CD41/CD33/CD45/CD117	CD41-FITC	IgG1	neat	5 µl	DAKO
	LAP: 41+/33+, 41+/117+	CD33-PE	IgG1	neat	5 µl	DAKO
		CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
10.	GPA/CD13/CD45/CD117	GPA-FITC	IgG1	neat	5 µl	DAKO
	LAP: GPA+/13+,	CD13-PE	IgG1	neat	5 µl	DAKO
	GPA+/117+, 13+/117+	CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC	IgG1	neat	5 µl	DAKO
11.	Izotype Ctrl.	IgG2a-FITC		neat	5 µl	BD
		IgG1-PE		neat	5 µl	DAKO
		CD45-PECy5	IgG1	neat	5 µl	DAKO
		IgG1-APC		neat	5 µl	DAKO
12.	HLA-	HLA-DR-	IgG2a	neat	5 µl	BD
	DR/CD33/CD45/CD117	FITC	IgG1	neat	5 µl	DAKO
	LAP: DR-/33+, DR+/33-,	CD33-PE	IgG1	neat	5 µl	DAKO
	DR-/117+, DR+/117-	CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD117-APC				
13.	Izotype Ctrl.	IgG2a-FITC		neat	5 µl	Serotec
		IgG1-PE		neat	5 µl	DAKO
		CD45-PECy5	IgG1	neat	5 µl	DAKO
		IgG1-APC		neat	5 µl	DAKO
14.	CD56/CD13/CD45/CD34	CD56-FITC	IgG2a	neat	5 µl	Serotec
	LAP: 56+/13+, 56+/34+	CD13-PE	IgG1	neat	5 µl	DAKO
		CD45-PECy5	IgG1	neat	5 µl	DAKO
		CD34-APC	IgG1	neat	5 µl	DAKO

Table 4.5. Four-colour FC antibody panels for detection of MRD in B-ALL, T-ALL and AML; antibodies used for set up of compensation parameters in B-ALL, T-ALL and AML.

15.	CD14/CD13/CD45/CD117 LAP: 14+/13+, 14+/117+	CD14-FITC CD13-PE CD45-PECy5 CD117-APC	IgG2a IgG1 IgG1 IgG1	neat neat neat neat	5 µl 5 µl 5 µl 5 µl	Serotec DAKO DAKO DAKO
16.	Izotype Ctrl.	IgM-FITC IgG1-PE CD45-PECy5 IgG1-APC	 IgG1 IgG1	neat neat neat neat	20 µl 5 µl 5 µl 5 µl	BC DAKO DAKO DAKO
17.	CDw65/CD13/CD45/CD34 LAP: w65+/13+, w65+/34+	CDw65-FITC CD13-PE CD45-PECy5 CD34-APC	IgM IgG1 IgG1 IgG1	neat neat neat neat	20 µl 5 µl 5 µl 5 µl	BC DAKO DAKO DAKO
18.	CD15/CD34/CD45/CD117 LAP: 15+/34+, 15+/117+	CD15-FITC CD34-PE CD45-PECy5 CD117-APC	IgM IgG1 IgG1 IgG1	neat neat neat neat	20 µl 5 µl 5 µl 5 µl	BC DAKO DAKO DAKO
19.	Izotype Ctrl.	IgG1-FITC IgG1-PE CD45-PECy5 IgG1-APC	 IgG1 	neat neat neat neat	5 µl 5 µl 5 µl 5 µl	BD DAKO DAKO DAKO
20.	CD7/CD13/CD45/CD117 LAP: 7+/13+, 7+/117+	CD7-FITC CD13-PE CD45-PECy5 CD117-APC	IgG1 IgG1 IgG1 IgG1	neat neat neat neat	5 µl 5 µl 5 µl 5 µl	BD DAKO DAKO DAKO
21.	CD38/CD34/CD45/CD117 LAP: quantitative	CD38-FITC CD34-PE CD45-PECy5 CD117-APC	IgG1 IgG1 IgG1 IgG1	neat neat neat neat	5 µl 5 µl 5 µl 5 µl	DAKO DAKO DAKO DAKO
22.	Izotype Ctrl.	IgG1-FITC IgG1-PE CD45-PECy5 IgG1-APC	 IgG1 	neat neat neat neat	5µl 20µl 5µl 5µl	DAKO BC DAKO DAKO
23.	CD13/m7.1/CD45/CD117 LAP: m7.1+	CD13-FITC m7.1-PE CD45-PECy5 CD117-APC	IgG1 IgG1 IgG1 IgG1	neat neat neat neat	5µl 20µl 5µl 5µl	DAKO BC DAKO DAKO

Compensations:

Tube	*Combination*	*Antibodies*	*Isotype*	*Dilution*	*Amount*	*Company*
C1.	Neg. Ctrl.	no				
C2.	Compenzation – FITC	CD45-FITC	IgG1	neat	5 µl	DAKO
C3.	Compenzation – PE	CD45-PE	IgG1	neat	5 µl	DAKO
C4.	Compenzation – PECy5	CD45-PECy5	IgG1	neat	5 µl	DAKO
C5.	Compenzation – APC	CD45-APC	IgG1	neat	5 µl	DAKO
C6.	Izotype Ctrl. - gating	IgG1-PECy5		neat	5 µl	DAKO

4.9.2. Antibody titration

The working amount/dilution of antibodies were optimised by antibody titration to prevent suboptimal staining or non-specific binding leading to false negative or positive results, respectively. The titrated antibody was diluted in PBS/BSA/SA in a 2-fold series and then used according to the protocols for surface, eventually intracellular, staining. The titration was carried out on bone marrow and peripheral blood cells, either normal or leukaemic, expressing the antigen of interest. In case of large quantitative differences in the antigen expression between normal and leukaemic cells (as in case of CD10), the titration was performed on both normal and leukaemic cells to ensure the correct amount of the antibody was chosen. Acquisition and analysis of the titration series on flow cytometer were carried out in the standard way. The mean fluorescence was then calculated for each amount/dilution of the antibody and the titration curve was constructed (Figure 4.1.). The optimal amount/dilution of the antibody was assessed from the standard curve as the one closest to but still below the maximum intensity plateau. Background fluorescence (fluorescence of cells not expressing the antigen) for this amount/dilution should not be greater that the isotype control. Specificity of the antibody was estimated according to the shape of the titration curve (in case of an antibody with higher unspecific binding the titration curve does not reach the plateau phase) (Protocol 8).

The amount or dilution of the relevant isotype control was determined from the protein content, so as the amount/concentration of the protein of the isotype control was equal to that of the antibody.

4.9.3. Staining for surface and intracellular antigens

The methods of staining for surface and intracellular antigens were developed according to the recommendations of the flow cytometer manufacturer (Becton Dickinson), and the antibodies and intracellular staining kits suppliers. The protocols were optimised for the best results (including the amount of antibodies and labelled cells, incubation times and temperatures, washing and blocking steps, lysing and permeabilising solutions used).

A method of whole blood lysis was adopted as it simplifies the procedures, decreases red blood cell and debris contamination and reduces unspecific antibody binding. Because the mononuclear cell separation of the patients' samples had to be performed for the molecular biological part of the study and the total amount of cells obtained often had to be split between flow cytometry and molecular biology, in some cases separated mononuclear cells from BM instead of whole bone marrow were used for FC. They were processed according to the same protocols and the results obtained were quite comparable with those of the whole bone marrow.

For intracellular staining, two commercially available kits for intracellular staining with the best referred performances were tested, IntraStain (DAKO) and Fix and Perm (Caltag). Both gave very similar results of a very good quality; IntraStain was then selected for the labelling as many of the antibodies used were also of DAKO origin.

Before the labelling procedure, the whole bone marrow, peripheral blood or separated mononuclears were diluted in CellWash/FBS to the WBC concentration of 1×10^7 / ml.

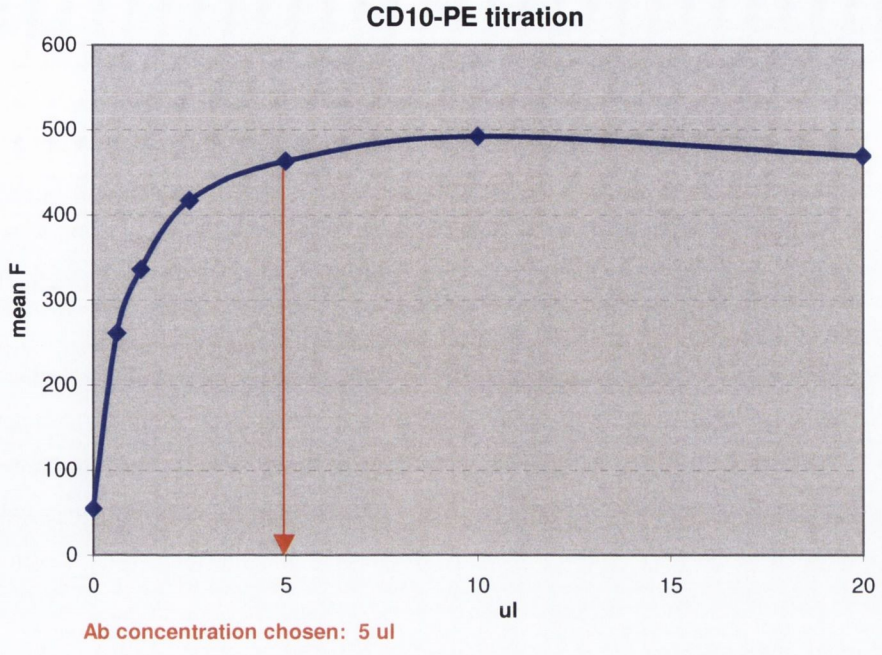


Figure 4.1. Titration curve of CD10-PE antibody (Ab – antibody).

In case of the staining for surface antigens, the combinations of antibodies were incubated with 100 µl of the sample for the optimised time, at room temperature in the dark. Following this, FACS Lysing solution (BD) was added and after further incubation the cells were centrifuged, washed in CellWash (BD) and fixed by resuspending in CellFix (BD). The sample was kept at 4 °C in dark till acquisition, and acquired on flow cytometer the same day, usually immediately (Protocol 9).

When staining for intracellular antigens was performed, 100 µl of the sample was first incubated with the antibodies against surface markers (like in the previous protocol), and washed in CellWash. The cells were then incubated with IntraStain Reagent A – Fixation, washed and consequently incubated with IntraStain Reagent B – Permeabilisation together with antibodies against intracellular antigens. All incubations took place at room temperature in the dark. Following another washing the sample was again fixed in CellFix and acquired on the flow cytometer immediately or stored at 4 °C in dark and acquired within 4 hours (Protocol 10).

At diagnosis, the patient's sample was labelled with the whole panel of antibody combinations as well as with the series necessary for compensation parameters set-up. After acquisition and analysis, the sample could be labelled with additional antibody combinations if necessary for better definition of LAPs and MRD.

The follow-up samples were labelled only with the antibody combinations chosen for MRD detection in the particular patient and corresponding isotype controls.

Control samples (normal and regenerating bone marrow) were labelled with the complete panels of antibodies.

4.9.4. Instrument and settings

All samples were acquired and analysed on FACSCalibur (Becton Dickinson) dual laser (Ar, He-Ne) flow cytometer. The machine was carefully maintained according to the recommended schedule, with daily and weekly cleaning procedures and thorough check-ups by specialist service technician in a 3-month period. Calibration of the machine was performed weakly using CaliBrite beads (BD) and FACSComp software (version 4.2., BD); it was always within the required limits.

In addition to that, individual compensation parameters were set up for every patient at diagnosis and used for all the following samples of this patient. The compensation parameters (to compensate for spectral overlap of different fluorochromes and their corresponding channels, and thus prevent false positive results) were set up with a series of patient's bone marrow cells labelled with single fluorochrome-conjugated antibodies (Table 4.5.) and by manual adjusting the compensation factors.

4.9.5. Acquisition of samples

Samples were run on FACSCalibur flow cytometer using CellQuest software (version 3.3., BD). Initially, 10 000 events were acquired to set the gates. Multiple gating strategy including "live" gating was adopted as this is more accurate in separation the target cell population from the remaining bone marrow cells and background, and thus decreases the risk of false positive results and improves the sensitivity. First gate, G1, was set around the cells of interest according to FSC/SSC properties. A second gate, G2, was set around the cells based on their expression of the main antigen characteristic for the leukaemia type, CD19/SSC for B-ALL, CD7/SSC for T-ALL and CD45/SSC for AML (Figure 4.2.). Additional gates were used in some cases to improve the discrimination between the region of possible presence of leukaemic cells and regions containing normal bone marrow and regenerating bone marrow cells only.

Only cells falling within all the defined gates were counted and considered for further analysis. For the immunotyping of the diagnostic sample, 10 000 of gated events were acquired. For MRD detection in the follow-up samples, 100 000 of gated events or the whole contents of the tube were acquired (whatever occurred first), corresponding to 300 000 – 900 000 of total events. Normal and regenerating control bone marrow samples were acquired in the same way as samples for MRD detection.

Special attention was paid to prevent cross-contamination of the samples and tubes. The detection probe of the machine was swiped with pieces of tissue and pure sheath fluid was run in between every tube; pure sheath fluid was also from time to time acquired to demonstrate there are no contaminating cells from the previous samples present.

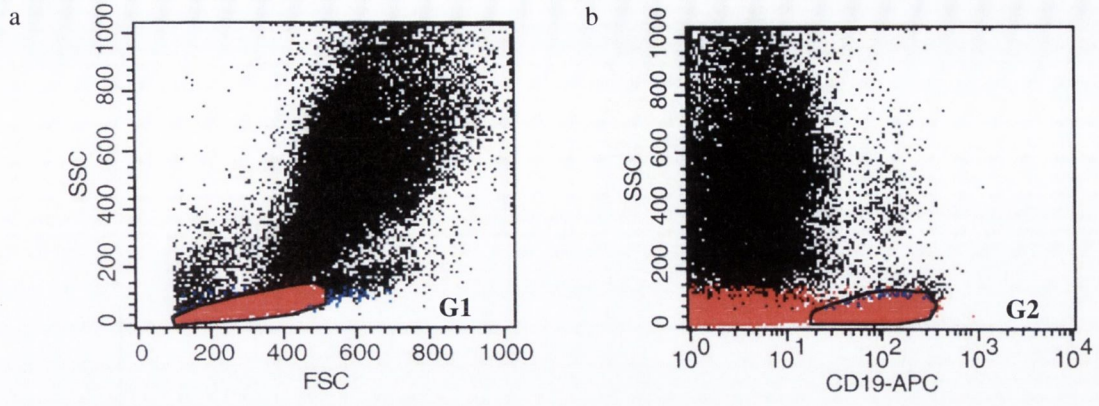


Figure 4.2. Multiple gating strategy used for FC acquisition and analysis. First gate G1 set around the population of lymphocytes and lymphoblasts based on their FSC/SCC properties (a), a second gate G2 set around the population of CD19 positive cells on the CD19/SSC plot (b) in a patient with B-ALL.

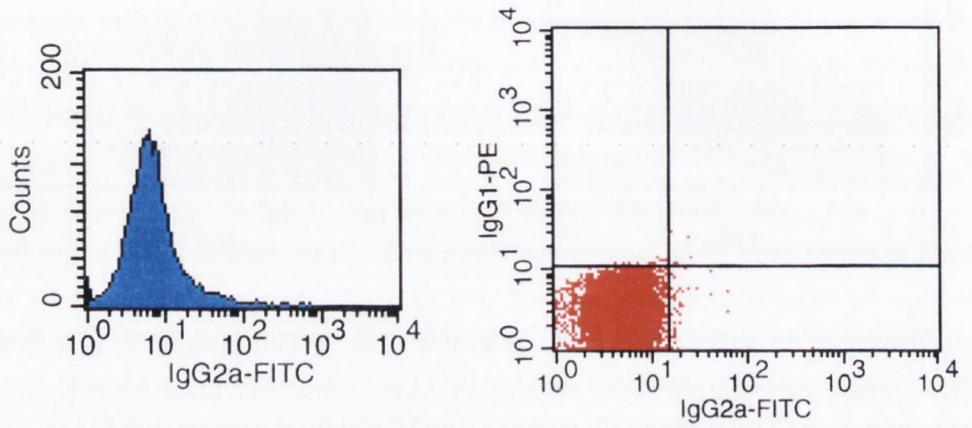
4.9.6. Analysis of samples, definition of LAPs and MRD

The acquired data were analysed using CellQuest software (version 3.3., BD). In a diagnostic sample, FSC/SSC and main antigen/SSC plots were created for the negative and isotype controls, gates G1, G2, eventually additional gates were set around the region of leukaemic blasts (similarly as in case of acquisition) and only cells falling within all the gates were further analysed (Figure 4.2.). Histograms displaying the expression profile of a single antigen and dot plots displaying the distribution and level of expression of two distinct antigens were plotted for all antibodies and pairs of antibodies of each antibody combination / each tube. Quadrants were set around the cells to define the limits of normal fluorescence (including autofluorescence and background fluorescence). This setting of plots, gates and quadrants was then saved and used for analysis of all the relevant LAPs in a sample (of the same isotype as the particular isotype control). Finally, a region was drawn around the group of cells expressing aberrant leukaemia associated phenotype and quadrant and region statistics were employed to calculate the amount of the cells of interest and their mean fluorescence (Figure 4.3.). For each patient, the setting of plots, gates and quadrants was kept and used for all the follow-up samples, occasionally minor changes were made to correct for the inter-assay variability.

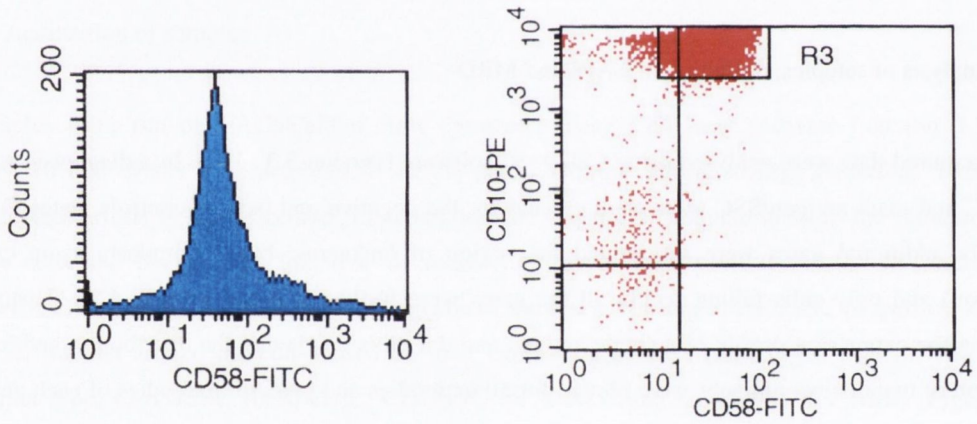
In the diagnostic sample, immunophenotyping was performed and LAPs suitable for further MRD detection identified. The leukaemic cell population was considered positive for a particular antigen or a combination of antigens defining a LAP, if ≥ 20 % of the cells expressed this antigen or LAP. Both qualitative LAPs (abnormal combinations of antigens) and quantitative LAPs (abnormal expression patterns or abnormal levels of expression) were detected. For each patient, at least 2 – 3 reliable LAPs were aimed to find and used for MRD detection in the follow-up samples.

In the follow-up samples, the presence and amount of cells expressing the chosen LAPs were detected and the MRD status and levels were quantified (Table 4.6.).

a



b



c

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	927	7.80	3.53	9.88	8.60	4898.09	2271.41
UR	10875	91.49	41.47	37.30	34.99	6356.30	6099.94
LL	79	0.66	0.30	5.86	5.20	5.82	5.02
LR	5	0.04	0.02	24.39	22.55	7.71	6.87

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	11886	100.00	45.32	34.95	30.96	6197.70	5371.86
R2	11886	100.00	45.32	34.95	30.96	6197.70	5371.86
R3	11315	95.20	43.14	36.20	33.43	6377.32	6177.06

Figure 4.3. Analysis of FC data. Isotype control (a), histogram (left) and dot plot (right); detection of LAPs (b), histogram (left) and dot plot (right) with a region drawn around the leukaemic cell population expressing an aberrant LAP (R3); and their quadrant and regional statistics (c).

Table 4.6. Definition of MRD presence and levels detected by FC.

MRD status and levels detected by FC:	
MRD negative	no cells with the defined LAP present
MRD positive	cells with the defined LAP present
very high level	≥ 10 % of cells with the LAP out of the total number of cells
high level	1 – 9.99 %
medium level	0.1 – 0.99 %
low level	0.01 – 0.09 %
very low level	< 0.01 %
MRD positivity > 0.01 % is recognised as prognostically significant	

4.9.7. Specificity and sensitivity tests

The specificity of the LAPs was assessed according to their presence, expression patterns and levels in the control samples – normal bone marrow and regenerating bone marrow from patients with other types of leukaemia, not expressing the particular abnormal immunophenotype or MRD negative by other method of MRD detection (PCR). LAPs with no or very low (< 0.01 % of total cells) expression in the control samples were preferentially chosen; however in some cases, especially in AML patients, also LAPs with expression < 0.1 % of total cells in control samples were used as no LAPs with higher specificity were available.

The sensitivity of the FC MRD detection was tested for particular LAPs in 10-fold dilution series (10^{-1} - 10^{-6}) of a positive sample diluted into a control sample (normal or regenerating bone marrow, or peripheral blood).

The specificity of LAPs in relation to their expression in control samples and sensitivity of the assay evaluated by the dilution series experiments defined the threshold for FC MRD detection.

4.10. Detection of chromosomal aberrations by RT-PCR and RQ-PCR

Detection of fusion gene transcripts from chromosome aberrations by RT-PCR and RQ-PCR for MRD detection were performed according to the BIOMED-1 Concerted Action (BIOMED-1) and Europe Against Cancer Program (EAC) recommendations and protocols [van der Velden *et al* 2003 and 2004a, van Dongen *et al* 1999, Gabert *et al* 2003, Beillard *et al* 2003] with respect to the latest knowledge in this area, methodology and results of published studies [Hilden *et al* 1997, Codrington *et al* 2000, Guerrasio *et al* 2002, van der Reijden *et al* 2002, Gameiro *et al* 2002, Elia *et al* 2003, Metzler *et al* 2004]. The principle of PCR and the detection of MRD by PCR of chromosomal aberrations are described in chapter 2.3.2.2.

4.10.1. Chromosomal aberrations detected

Of a number of chromosomal alterations found in childhood acute leukaemia, the standardised PCR assays were developed for a group of alterations that occur with higher frequencies and are thus suitable for MRD monitoring.

The following chromosomal aberrations were used for MRD detection: t(9;22) *BCR-ABL* p190 and p210, t(12;21) *TEL-AML1*, t(1;19) *E2A-PBX1* and t(4;11) *MLL-AF4* in B-ALL; microdel(1p) *SIL-TAL1* in T-ALL; t(15;17) *PML-RARA*, t(8;21) *AML1-ETO* and inv(16) *CBFB-MYH11* in AML (see Table 2.21.)

4.10.2. RT reaction

The cDNA synthesis was performed according to the EAC recommendations, starting from 1 µg of total RNA.

RNA extracted from cells was first quantified by spectrophotometry (SPEC) and checked for purity and integrity by calculating the ratio of absorbance at 260/280 nm and by electrophoresis on 2 % agarose gel (Protocol 11 and 6, Figure 4.4.) In case of very low concentration, the RNA was concentrated in a heated vacuum centrifuge.

The starting 1 µg of RNA was diluted in sdH_2O and initially denaturated by heating up with consequent chilling on ice. RT master mix containing random primers, dNTPs, 5X First strand buffer, DTT, RNasin and SuperScript II was prepared and added to the RNA. to a final volume of 20 µl. After pre-incubation (activation of random primers), the RT reaction was performed at 42°C for 45 min, ended by heat inactivation of the enzymes followed by cooling down to 4°C. For RQ-PCR, the product of RT was then diluted with 30 µl of sdH_2O. The synthesised cDNA was stored at - 20°C (Protocol 12).

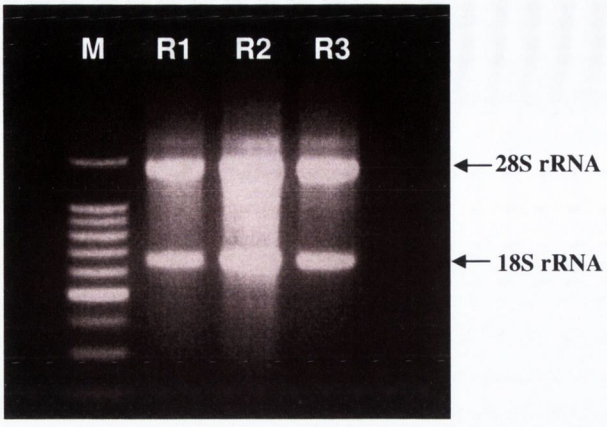


Figure 4.4. RNA purity and integrity checking. Electrophoresis of 3 RNA samples on agarose gel showing sharp clear 28S and 18S rRNA bands (M - size marker).

4.10.3. RT-PCR

The RT-PCR reactions for chromosomal aberrations were performed according to BIOMED-1 protocols. For each chromosomal aberration 3 RT-PCR reactions were set up: primary (A+B), confirmatory (C+E or E+D) and nested (A+B → C+D). In case of SIL-TAL1, seminested instead of nested reaction was employed. The sets of primers used for the PCR reactions, their sequences, localisation within the fusion genes and the sizes of generated PCR products are described in detail in the BIOMED-1 protocols [van Dongen *et al* 1999] (Table 4.7.). The reactions were optimised by magnesium titration, and in some cases by addition of solutions enhancing the PCR reactions (DMSO) (see below).

The PCR master mix containing forward and reverse primers, dU/dNTPs, 10X PCR Gold buffer, $MgCl_2$, DMSO in some cases, AmpliTaq Gold DNA polymerase (a hot start DNA polymerase), Uracil-N-Glycosylase heat labile (in first round PCR only) and sdH_2O to make a final volume of the reaction 50 µl was prepared. For first round PCR – primary or confirmatory – 2 µl of cDNA (10 % of the RT product) were added per one reaction, for nested PCR 1 µl of DNA (2 % of the first round PCR product) was used per one reaction. The RT-PCR was performed on PTC-200 (MJ Research). After pre-incubation (UNG step) and heat activation of AmpliTaq enzyme, 35 cycles of the PCR reaction were run (Protocol 13). The PCR product was either stored at – 20 °C or immediately run on the agarose gel.

PCR products were analysed by electrophoresis on 2 % agarose gel with ethidium bromide and visualised under UV light (Protocol 14). Results were considered positive if a clear band of the appropriate size was present.

All diagnostic samples were screened for the relevant chromosomal aberrations by the primary RT-PCR reaction. Positive samples were then tested again by confirmatory RT-PCR. In case of very low amount of RNA obtained from a diagnostic sample, nested PCR could be performed. Nested PCR may also be used to verify negative results of RQ-PCR.

Table 4.7. Primers used for RT-PCR of chromosomal aberrations in acute leukaemia (according to BIOMED-1 protocol) - cont.

RT-PCR primers for detection of chromosomal aberrations in acute leukaemia			
Chromosomal aberration	Primer code	(size)	Sequence 5' - 3'
t(12;21) TEL-AML1	TEL-A	(20)	TGC ACC CTC TGA TCC TGA AC
	AML1-B	(19)	AAC GCC TCG CTC ATC TTG C
	TEL-C	(22)	AAG CCC ATC AAC CTC TCT CAT C
	AML1-D	(18)	TGG AAG GCG GCG TGA AGC
	TEL-E5'	(20)	CGC ACC AGG AGA ACA ACC AC
t(1;19) E2A-PBX1	E2A-A	(19)	CAC CAG CCT CAT GCA CAA C
	PBX1-B	(19)	TCG CAG GAG ATT CAT CAC G
	E2A-C	(19)	CAC CCT CCC TGA CCT GTC T
	PBX1-D	(19)	GGC CTG CTC GTA TTT CTC C
	PBX1-E3'	(19)	TGA ACT TGC GGT GGA TGA T
t(4;11) MLL-AF4	MLL-A	(17)	CCG CCT CAG CCA CCT AC
	AF4-B	(20)	TGT CAC TGA GCT GAA GGT CG
	MLL-C	(18)	AGG ACC GCC AAG AAA AGA
	AF4-D	(20)	CGT TCC TTG CTG AGA ATT TG
	MLL-E5'	(18)	AAG CCC GTC GAG GAA AAG
t(9;22) BCR-ABL p190 and p210	BCR-e1-A (p190)	(21)	GAC TGC AGC TCC AAT GAG AAC
	BCR-b1-A (p210)	(22)	GAA GTG TTT CAG AAG CTT CTC C
	ABL-a3-B	(21)	GTT TGG GCT TCA CAC CAT TCC
	BCR-e1-C (p190)	(21)	CAG AAC TCG CAA CAG TCC TTC
	BCR-b2-C (p210)	(21)	CAG ATG CTG ACC AAC TCG TGT
	ABL-a3-D	(23)	TTC CCC ATT GTG ATT ATA GCC TA
	ABL-a3-E3'	(23)	TGA CTG GCG TGA TGT AGT TGC TT
microdel(1p) SIL-TAL1	SIL-A	(19)	TCC CGC TCC TAC CCT GCA A
	TAL1-B	(18)	CGC GCC CAG TTC GAT GAC
	TAL1-C	(18)	CCG CGT CCC GTC CCT CTA
	TAL1-E3'	(19)	CGT CGC GGC CCT TTA AGT C
t(15;17) PML-RARA	PML-A1 (bcr1,2)	(21)	CAG TGT ACG CCT TCT CCA TCA
	PML-A2 (bcr3)	(18)	CTG CTG GAG GCT GTG GAC
	RARA-B	(20)	GCT TGT AGA TGC GGG GTA GA
	PML-C1 (bcr1,2)	(21)	TCA AGA TGG AGT CTG AGG AGG
	PML-C2 (bcr3)	(19)	AGC GCG ACT ACG AGG AGA T
	RARA-D	(20)	CTG CTG CTC TGG GTC TCA AT
	RARA-E3'	(20)	GCC CAC TTC AAA GCA CTT CT

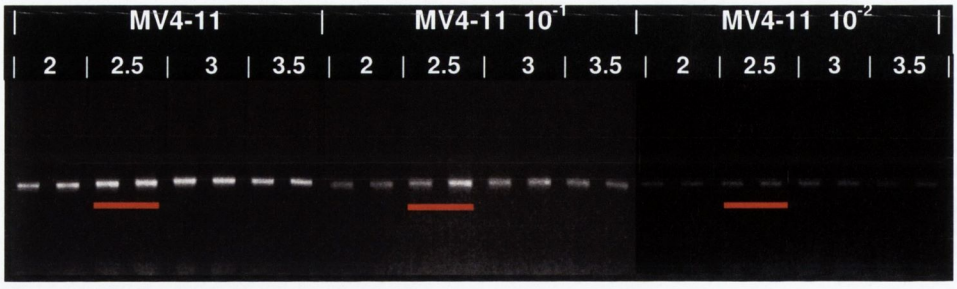
Table 4.7. Primers used for RT-PCR of chromosomal aberrations in acute leukaemia (according to BIOMED-1 protocol).

Chromosomal aberration	Primer code	(size)	Sequence 5' - 3'
t(8;21) AML1-ETO	AML1-A	(21)	CTA CCG CAG CCA TGA AGA ACC
	ETO-B	(21)	AGA GGA AGG CCC ATT GCT GAA
	AML1-C	(22)	ATG ACC TCA GGT TTG TCG GTC G
	ETO-D	(22)	TGA ACT GGT TCT TGG AGC TCC T
	AML1-E5'	(24)	TGG CTG GCA ATG ATG AAA ACT ACT
inv(16p) CBFB-MYH11	CBFB-A	(22)	GCA GGC AAG GTA TAT TTG AAG G
	MYH11-B1 (D,E,G,H)	(20)	TGA AGC AAC TCC TGG GTG TC
	MYH11-B2 (A,B,C,F,J)	(22)	TCC TCT TCT CCT CAT TCT GCT C
	CBFB-C	(20)	GGG CTG TCT GGA GTT TGA TG
	MYH11-D1 (D,E,G,H)	(20)	TCC CTG TGA CGC TCT CAA CT
	MYH11-D2 (A,B,C,F,J)	(18)	CTT GAG CGC CTG CAT GTT
	CBFB-E5'	(21)	CAG GGA GAA CAG CGA CAA ACA

4.10.4. Magnesium and DMSO titration for RT-PCR

All the PCR reactions were optimised by $MgCl_2$ titration. This was carried out using RNA extracted from cell lines expressing the target chromosomal aberration (see Table 4.1.) and 10^{-1} and 10^{-2} dilutions of this positive RNA in negative RNA of other cell line. As negative controls, RNA from cell line not expressing the aberration and no template control (sdH_2O) were used. The RT step was performed in a standard way. PCR master mixes containing $MgCl_2$ in concentration 0 – 6 mM were prepared and the PCR reactions were set in duplicates for each $MgCl_2$ concentration. The $MgCl_2$ concentration giving the strongest and clearest bands on the agarose gel analysis was then selected for further use (Protocol 15 and Figure 4.5.). In vast majority of the reactions the chosen $MgCl_2$ concentration was between 2.0 – 3.5 mM; only in nested PCR for *MLL-AF4*, a higher $MgCl_2$ concentration (4.5 mM) was necessary.

In case of PCR reactions for *E2A-PBX1*, *TEL-AML1* and *SIL-TAL1*, even the optimal $MgCl_2$ concentration did not produce very clear bands or enough good sensitivity of the reactions. Addition of some of the PCR enhancing agents was therefore tested (DMSO, BSA, trehalose, glycerol). DMSO worked best in all cases and its optimal concentration was then titrated in a similar way as $MgCl_2$. The optimal concentration of DMSO appeared to be 2 % for *E2A-PBX1* and *TEL-AML1* (all sets of primers) and 5 % for *SIL-TAL1* (all sets of primers).



$MgCl_2$ concentration chosen: 2.5 mM

Figure 4.5. Magnesium titration for t(4;11) MLL-AF4 primary PCR reaction (primers A + B), using MV4-11 cell line and its 10^{-1} and 10^{-2} dilutions. Comparative electrophoresis of products of PCR reactions containing 2 – 3.5 mM of $MgCl_2$.

4.10.5. RQ-PCR

The RQ-PCR of fusion gene transcripts from chromosomal aberrations was performed according to the EAC protocols. For detection of each aberration, 1 – 3 sets of primers and a 6FAM-TAMRA labelled probe were used. Together with the target fusion gene (FG), a control house-keeping gene (CG), either Abelson (*ABL*), β-2-microglobulin (*B2M*) or β-glucuronidase (*GUS*), was always amplified to enable corrections for the starting amount and quality of RNA. Plasmid DNA calibrators in 10 log dilutions (10^1 – 10^6) were used for absolute quantification of the fusion and control gene expression. The sets of primers and probes employed in the RQ-PCR reactions, their sequences and localisation within the genes are described in detail in the EAC protocols [Gabert *et al* 2003, Beillard *et al* 2003] (Table 4.8.). Standardised plasmid DNA calibrators were purchased (Ipsogen).

The RQ-PCR master mix containing forward and reverse primers, probe, 2X TaqMan Universal Master Mix and sdH_2O to the final volume of the reaction of 25 µl was prepared. cDNA 5 µl (10 % of the diluted RT product) or 5 µl of plasmid DNA calibrators (log 10 copies) were added into one reaction. Reactions for the fusion gene were carried out in triplicate, reactions for the control gene and for calibrators in duplicate. The RQ-PCR was performed on ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After pre-incubation and heat activation of AmpliTaq enzyme, 50 cycles were run (Protocol 16).

The results of RQ-PCR were analysed by ABI PRISM 7700 Sequence Detection System software (version 1.6) and Microsoft Excel software. The threshold was set at 0.1 except for *PML-RARA* (0.05), the base line was set between cycles 3 – 15, except for *B2M* (3 – 10). Based on the plasmid calibrators, standard curves for the fusion and control genes were created and their slope, intercept and correlation coefficient evaluated. A sample was considered positive if at least one of the triplicate or duplicate RQ-PCR reactions was positive. For each sample, mean Ct ± standard deviation and mean ΔCt ± standard deviation (mean Ct [FG] - mean Ct [CG]) were calculated. The fusion gene copy number (FGCN), control gene copy number (CGCN) and FG normalized copy number (NCN = FGCN / CGCN) were

calculated according to the standard curve method (Bulletin 2, 10/2001, Applied Biosystems). The sensitivity levels of the individual experiments may be assessed according to the EAC recommendations (sensitivity = $-\log_{10}$ [NCN] – $\log_{10}$ [CGCN]).

Table 4.8. Primers and probes used for RQ-PCR of fusion gene transcripts in chromosomal aberrations and control genes in acute leukaemia (according to EAC protocol) - cont.

RQ-PCR primers and probes for detection of fusion gene transcripts in chromosomal aberrations and control genes in acute leukaemia			
Chromosomal aberration	Primer/probe code	(size)	Sequence 5' - 3'
t(12;21) TEL-AML1	ENF301	(19)	CTC TGT CTC CCC GCC TGA A
	ENPr341	(20)	TCC CAA TGG GCA TGG CGT GC
	ENR361	(16)	CGG CTC GTG CTG GCA T
t(1;19) E2A-PBX1	ENF101	(19)	CCA GCC TCA TGC ACA ACC A
	ENP141	(22)	CCC TCC CTG ACC TGT CTC GGC C
	ENR161	(21)	GGG CTC CTC GGA TAC TCA AAA
t(4;11) MLL-AF4	ENF207 (e9-e4,5)	(25)	CCC AAG TAT CCC TGT AAA ACA AAA A
	ENF208 (e10,11-e4,5)	(23)	GAT GGA GTC CAC AGG ATC AGA GT
	ENP242	(22)	CAT GGC CGC CTC CTT TGA CAG C
	ENR262	(22)	GAA AGG AAA CTT GGA TGG CTC A
t(9;22) BCR-ABL p190 and p210	ENF402 (p190)	(18)	CTG GCC CAA CGA TGG CGA
	ENF501 (p210)	(21)	TCC GCT GAC CAT CAA YAA GGA*)
	ENP541	(25)	CCC TTC AGC GGC CAG TAG CAT CTG A
	ENR561	(21)	CAC TCA GAC CCT GAG GCT CAA
microdel(1p) SIL-TAL1	ENF601	(19)	CGC TCC TAC CCT GCA AAC A
	ENP641	(22)	ACC TCA GCT CCG CGG AAG TTG C
	ENR664	(19)	CCG AGG AAG AGG ATG CAC A
t(15;17) PML-RARA	ENF903 (bcr1)	(19)	TCT TCC TGC CCA ACA GCA A
	ENF906 (bcr2)	(19)	ACC TGG ATG GAC CGC CTA G
	ENF905 (bcr3)	(19)	CCG ATG GCT TCG ACG AGT T
	ENP942	(20)	AGT GCC CAG CCC TCC CTC GC
	ENR962	(21)	GCT TGT AGA TGC GGG GTA GAG
t(8;21) AML1-ETO	ENF701	(22)	CAC CTA CCA CAG AGC CAT CAA A
	ENP747	(30)	AAC CTC GAA ATC GTA CTG AGA AGC ACT CCA
	ENR761	(22)	ATC CAC AGG TGA GTC TGG CAT T
inv(16)(p13;q22) CBFB-MYH11	ENF803	(22)	CAT TAG CAC AAC AGG CCT TTG A
	ENPr843	(22)	TCG CGT GTC CTT CTC CGA GCC T
	ENR862 (type A)	(17)	AGG GCC CGC TTG GAC TT
	ENR863 (type D)	(21)	CCT CGT TAA GCA TCC CTG TGA
	ENR865 (type E)	(23)	CTC TTT CTC CAG CGT CTG CTT AT

Table 4.8. Primers and probes used for RQ-PCR of fusion gene transcripts in chromosomal aberrations and control genes in acute leukaemia (according to EAC protocol).

Control gene	Primer code	(size)	Sequence 5' - 3'
Abelson (ABL)	ENF1003	(31)	TGG AGA TAA CAC TCT AAG CAT AAC TAA AGG T
	ENPr1043	(28)	CCA TTT TTG GTT TGG GCT TCA CAC CAT T
	ENR1063	(21)	GAT GTA GTT GCT TGG GAC CCA
β-2-microglobulin (B2M)	ENF1302	(18)	GAG TAT GCC TGC CGT GTG
	ENPr1342	(26)	CCT CCA TGA TGC TGC TTA CAT GTC TC
	ENR1362	(18)	AAT CCA AAT GCG GCA TCT
β-glucuronidase (GUS)	ENF1102	(26)	GAA AAT ATG TGG TTG GAG AGC TCA TT
	ENPr1142	(26)	CCA GCA CTC TCG TCG GTG ACT GTT CA
	ENR1162	(22)	CCG AGT GAA GAT CCC CTT TTT A

(ENF – European network forward primer, ENP – European network TaqMan probe, ENPr – European network TaqMan reverse probe, ENR – European network reverse primer; *) – cytidine or thymidine appears on the BCR p210 primer according to the recently described polymorphism)

The RQ-PCR detection and quantification of fusion gene transcripts for MRD monitoring was performed in the patients with chromosomal aberrations confirmed by RT-PCR. The diagnostic and all the follow-up samples of each of these patients were analysed by RQ-PCR, preferably together on one TaqMan plate. The MRD status and levels were defined according to the fusion gene NCN detected (Table 4.9.). Negative results may be confirmed by nested RT-PCR, particularly in cases where nested PCR shows higher sensitivity than RQ-PCR.

Table 4.9. Definition of MRD presence and levels detected by PCR of chromosomal aberrations.

MRD status and levels detected by PCR of chromosomal aberrations:	
MRD negative	all reactions for the FG negative
MRD positive	at least one of the reactions for the FG positive
very high level	NCN (FGCN / CGCN) $\geq 10^{-1}$
high level	$10^{-1} - 10^{-2}$
medium level	$10^{-2} - 10^{-3}$
low level	$10^{-3} - 10^{-4}$
very low level	$< 10^{-4}$
MRD positivity > 10^{-4} is recognised as prognostically significant	

4.10.6. Sensitivity and specificity tests

The specificity of the RT-PCR and RQ-PCR reactions was assessed from positive controls (cell line or patient's RNA containing the particular chromosomal aberration) and negative controls (cell line or patient's or normal donor's RNA lacking the particular aberration) that were always included and analysed together with the tested samples.

The sensitivity of the RT-PCR and RQ-PCR reactions was evaluated in a 10-fold dilution series (10^{-1} - 10^{-6}) of positive cell line or patient's RNA diluted into negative cell line or donor's RNA (Protocol 17). For RQ-PCR, the sensitivity was also determined from the plasmid calibrator series.

As the specificity of PCR for chromosomal aberrations is in general very high, the sensitivity established from the RNA dilution series and plasmid calibrators defined the threshold of MRD detection.

4.11. Detection of clonal IG/TCR rearrangements by PCR and RQ-PCR

Detection of clonal *IG/TCR* rearrangements by PCR and RQ-PCR for MRD detection was performed according to the BIOMED-1 and BIOMED-2 Concerted Action (BIOMED-1, BIOMED-2) recommendations and protocols [Langerak *et al* 1997, Pongers-Willemse *et al* 1998 and 1999, van der Velden *et al* 2003, van Dongen *et al* 2003], and according to latest knowledge, methodology and results of recently published studies [Langerak *et al* 2001, van der Velden *et al* 2002b and c, Bruggemann *et al* 2000 and 2004, Verhagen *et al* 2000, Szczepanski *et al* 2000, 2001b and 2002a and b, Tumkaya *et al* 2001, Li *et al* 2001, de Haas *et al* 2001, Boeckx *et al* 2002, Pine *et al* 2003, Germano *et al* 2003]. The principle of PCR and the detection of MRD by PCR of clonal *IG/TCR* rearrangements are described in chapter 2.4.2.2. This analysis was performed in ALL patients only.

4.11.1. PCR screening of IG/TCR clonality

The screening of diagnostic ALL samples for clonal *IG/TCR* rearrangements was performed using multiplex gene clonality assays (InVivoScribe) and protocols recommended by BIOMED-2. The combinations of primers used in the multiplex reactions, their sequences, localisation within the genes and the sizes of generated PCR products are described in detail in the BIOMED-2 protocols [van Dongen *et al* 2003] (Table 4.10.). Appropriate types of assay kits for gel detection or fluorescence detection were used. B-ALL samples were initially screened for *IGH* and *IGK*, T-ALL samples for *TCRD* and *TCRG*. In both cases, other assays could be used if the initial ones do not generate good results and suitable targets for MRD monitoring (eg. *TCRG* and *TCRD* in B-ALL, *TCRB* in T-ALL).

The DNA from the patient's diagnostic sample was diluted to a starting concentration 100 ng / µl. AmpliTaq Gold DNA polymerase and Uracil-N-Glycosylase heat labile were added to the pre-developed master mixes. Into one reaction of the final volume 25 µl, 100 – 200 ng of DNA was added. Positive controls (positive DNA) and negative controls (human polyclonal genomic DNA, sdH_2O) were included for each multiplex clonality assay. Every sample was tested with all the relevant clonality assay reactions

and also with Specimen control size ladder pre-developed assay to ensure the DNA quantity and quality was sufficient to yield valid results.

PCR was performed on PTC-200 (MJ Research). After pre-incubation (UNG step) and heat activation of AmpliTaq enzyme, 35 cycles of the PCR reaction were carried out. The PCR product was further analysed and stored at – 20 °C (Protocol 18).

Table 4.10. PCR IG/TCR clonality screening with multiplex reactions.

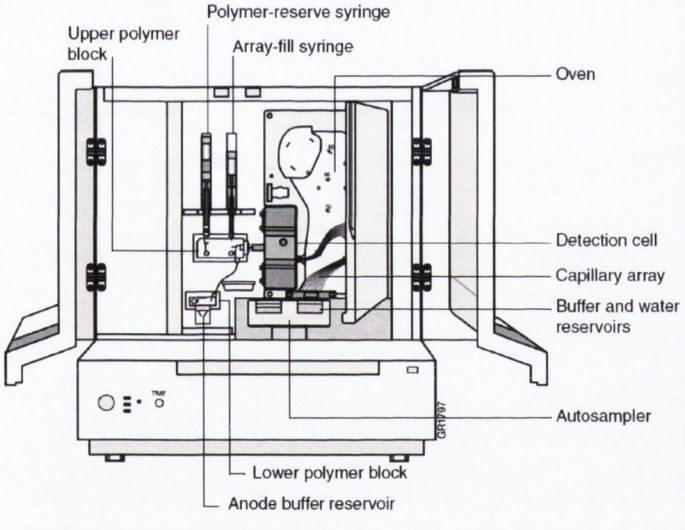
PCR IG/TCR clonality screening with multiplex assays [van Dongen 2003]			
gene	tube	Fwd primers	Rvs primers
IGH	A	VH1-FR1, VH2-FR1, VH3-FR1, VH4-FR1, VH5-FR1, VH6-FR1	JH consensus
	B	VH1-FR2, VH2-FR2, VH3-FR2, VH4-FR2, VH5-FR2, VH6-FR2, VH7-FR2	JH consensus
	C	VH1-FR3, VH2-FR3, VH3-FR3, VH4-FR3, VH5-FR3, VH6-FR3, VH7-FR3	JH consensus
	D	DH1, DH2, DH3, DH4, DH5, DH6	JH consensus
	E	DH7	JH consensus
IGK	A	Vk1f/6, Vk2f, Vk3f, Vk4, Vk5, Vk7	Jk1-4, Jk5
	B	Vk1f/6, Vk2f, Vk3f, Vk4, Vk5, Vk7, INTR	Kde
TCRB	A	Vb2, Vb4, Vb5/1, Vb6a/11, Vb6b/25, Vb6c, Vb7, Vb8a, Vb9, Vb10, Vb11, Vb12a/3/13a/15, Vb13b, Vb13c/12b/14, Vb16, Vb17, Vb18, Vb19, Vb20, Vb21, Vb22, Vb23/8b, Vb14	Jb1.1, Jb1.2, Jb1.3, Jb1.4, Jb1.5, Jb1.6, Jb2.2, Jb2.6, Jb2.7
	B	Vb2, Vb4, Vb5/1, Vb6a/11, Vb6b/25, Vb6c, Vb7, Vb8a, Vb9, Vb10, Vb11, Vb12a/3/13a/15, Vb13b, Vb13c/12b/14, Vb16, Vb17, Vb18, Vb19, Vb20, Vb21, Vb22, Vb23/8b, Vb14	Jb2.1, Jb2.3, Jb2.4, Jb2.5
	C	Db1, Db2	Jb1.1, Jb1.2, Jb1.3, Jb1.4, Jb1.5, Jb1.6, Jb2.1, Jb2.2, Jb2.3, Jb2.4, Jb2.5, Jb2.6, Jb2.7
TCRG	A	VgIf, Vg10	Jg1.1/2.1, Jg1.3/2.3
	B	Vg9, Vg11	Jg1.1/2.1, Jg1.3/2.3
TCRD		Vd1, Vd2, Vd3, Vd4, Vd5, Vd6, Dd2	Jd1, Jd2, Jd3, Jd4, Dd3

Detection of the presence of monoclonal products was performer either by electrophoresis on high-resolution agarose gels or by capillary electrophoresis (GeneScan).

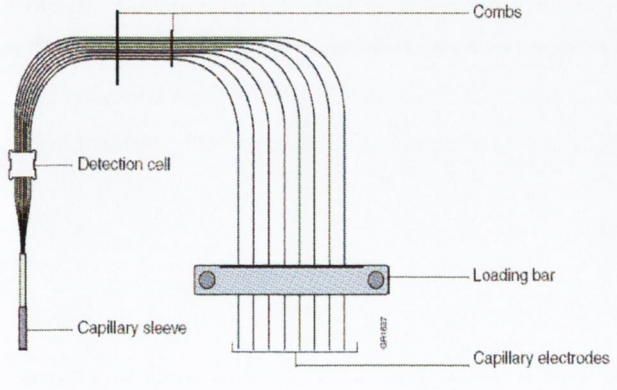
In case of gel detection, PCR products were analysed by electrophoresis on 2 % MetaPhore agarose/TBE gel with ethidium bromide and visualised under UV light. Results were considered positive if one or several clear band(s) of the appropriate size were present. Only reaction(s) giving a single band, corresponding to a monoclonal population, were selected for the further tests. If none of the reactions generated a single band and only multiple bands were present indicating possible biallelic rearrangements, bi- or oligoclonality, following options were considered: screening the sample with further multiplex assays, re-screening the sample with singleplex reactions (Protocol 21), separate cutting off the bands from the gel and their purification.

For GeneScan analysis the primers in the multiplex clonality assay pre-developed master-mixes are conjugated with different fluorescent dyes. The PCR was carried out as described previously. The PCR product was added to Hi-Di Formamide containing ROX size standards and this mixture was then applied to an optical reaction plate, denatured by heating to 95 °C followed by a quick chill on ice, and run on ABI3100 Genetic Analyzer machine (Figure 4.6.). The settings of the machine were adjusted as recommended in In VivoScribe protocols (IGH Gene Clonality Assay IVS) (Protocol 20). The results were analysed using GeneMapper (version 3.7) software. Results were considered positive if one or more clearly monoclonal peak(s) of the appropriate size were present. Only reaction(s) giving a single monoclonal peak, corresponding to a monoclonal population, were selected for the further tests. In case of suspected presence of bi- or oligoclonal population, following options were considered: screening the sample with further multiplex assays, re-screening the sample with singleplex reactions (Protocol 21), or running the sample on MetaPhore agarose gel and separate cutting off the corresponding bands and their purification.

ABI 3100 Genetic Analyzer – front view, doors open



Capillary array



The ABI 3100 Genetic Analyzer is an automated, high-throughput, capillary electrophoresis system for analysis of fluorescently labelled DNA fragments. The system can accurately separate a mixture of DNA fragments according to their lengths and determines the order of the four deoxyribonucleotide bases (sequencing analysis) or the lenght of each fragment and its relative concentration (fragment analysis, genescan).

The system consists of an autosampler, capillary array of various lenght (36 cm for fragment analysis and rapid sequencing of medium-size DNA fragments, 50 cm for standard sequencing) containing 16 fused-silica capillaries, detection cell containing laser and CCD camera, and computer workstation.

The samples containing fluorescently labelled DNA fragments are prepared in optical reaction plates and placed on the autosampler of the instrument. Capillaries of the capillary array are filled with a flowable polymer 3100 POP6 or POP4, a medium that separates the DNA fragments. The autosampler positions the 16 capillaries into the sample wells. The fluorescently labelled DNA is loaded into the capillaries by a short period of electrophoresis called electrokinetic injection. The capillaries are rinsed with water to remove sample adhering to capillary sides. The autosampler then moves and poistions the capillaries into the buffer chamber filled with 1X EDTA buffer for electrophoresis. During electrophoresis, heat is evenly and efficiently dissipated from the surface, which enables higher voltage to be applied across all capilaries without loss of resolution. When the DNA fragments reach the detection cell, they move through the path of a laser beam that excites the dye molecules. The fluorescence emitted from the samples is collected, spectrally separated by a reflective spectrograph and focused as columns of ligh onto the charge-coupled device (CCD) that is a part of the CCD camera. This camera converts the fluorescence into electronic information that is processed and interpreted by the data collection software. The results are dispayed in plots (electropherogram). (ABI PRISM 3100 Genetic Analyzer User's Manual, Applied Biosystems)

(pictures reproduced from the user's manual)

Figure 4.6. ABI 3100 genetic analyzer used for genescanning and sequencing analysis.

4.11.2. Purification of PCR products

The PCR products were cleaned up using the Qiagen QIAquick PCR purification and QIAquick gel extraction kits to remove enzymes and other components that could interfere with further applications. DNA is isolated from the PCR mix or agarose gel by adsorption to the silica-gel membrane of spin columns while contaminants pass through the column. Binding buffers in the kits provide the correct salt concentration and pH for binding of DNA to the membrane – the adsorption of nucleic acids to silica-gel surfaces occurs only in the presence of high concentration of chaotropic salts and low pH. Buffer PB in the PCR purification kit allows binding of PCR products ≥ 100 bp while removing primers up to 40 nucleotides; buffer QC in the gel extraction kit also solubilises the agarose gel. During washing, impurities are removed. The pure DNA is then eluted with Tris buffer or water (low-salt solutions).

The cleaning of PCR products and gel extracts was performed according to the QIAquick Spin Handbook (Qiagen, July 2002). Cleaned DNA was stored at -20 °C (Protocol 22 and 23).

4.11.3. Sequencing of the cleaned PCR products

The sequencing reaction was set up using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The principle is a chain termination method (Sanger) employing chain terminating dideoxynucleotides labelled with fluorescent dyes (four different fluorochromes for the four different nucleotides). These are present in low concentration in the PCR mix and after their incorporation by DNA polymerase they interrupt DNA synthesis resulting in a series of related DNA fragments. The fragments are size-separated by electrophoresis and the nucleotide order of the original template is inferred from the detected fluorescence of the fragments.

Master mixes, containing primer(s), Cycle Sequencing Mix and sdH_2O to the final volume of the reaction 20 µl were prepared, and 5 µl of the DNA template (cleaned PCR product) per reaction was added. The primers used for sequencing were the same as in the previous IG/TCR PCR screening [van Dongen *et al* 2003]. If the PCR product was a result of a multiplex assay, a mixture of all forward primers and a mixture of all reverse primers employed in the assay were prepared and used. For each sample, two separate sequencing reactions were carried, one with forward and one with reverse primer(s). pGEM plasmid and M13 fwd primer for its detection, both included in the kit, were used as a positive control.

The sequencing reaction was performed on GeneAmp 2400 thermal cycler (Applied Biosystems). After heat activation, 25 cycles of the reaction were run (Protocol 24). The sequencing product was cleaned or stored before that at – 20 °C.

4.11.4. Purification of the sequencing reaction

The products of the sequencing reaction were cleaned up using Qiagen DyeEx 2.0 Spin kit to remove unincorporated dye terminators that could otherwise interfere with the detection of the sequencing products.

The procedure uses gel-filtration technology and was performed according to the recommended protocol (DyeEx Handbook, Qiagen, May 2002). The sequencing products were applied to gel-filtration resin in microspin columns and centrifugated at the recommended g (Protocol 25).

The cleaned recovered products were dried in heated vacuum centrifuge and either stored at – 20 °C or run on the sequencer.

4.11.5. Electrophoresis of the cleaned sequencing products and sequence analysis

The dry sequencing products were resuspended in HiDi formamide and run on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The results were then analysed using SeqMan II (version 5.0) DNASTAR software to obtain the clonal sequence of the *IG/TCR* region (Protocol 26).

4.11.6. Examination of the clonal sequence and design of allele-specific oligonucleotide (ASO) primers

The clonal sequence was compared with the main sequence databases (IG BLAST: www.ncbi.nlm.nih.gov/igblast, V BASE: www.mrc-cpe.cam.ac.uk/DNAPLOT.php?menu=901, V QUEST: http://imgt.cines.fr/textes/vquest) to identify the V, D and J segments, and the clone-specific junctional regions joining them. Allele-specific oligonucleotide (ASO) primers were then designed within these junctional regions by checking the sequences manually or using PrimerExpress 2.0 software. If the length and sequence of the junctional region allowed, 2 forward primers were selected for each clone-specific junctional region (Protocol 26).

4.11.7. ASO primers testing

The ASO primers were tested for efficiency, specificity and sensitivity in RQ-PCR reaction. A 10-fold dilution series of patient's diagnostic DNA into human polyclonal genomic DNA was prepared and the polyclonal DNA was also used as a negative control (Protocol 27). Later, due to shortage of polyclonal human DNA, patients' samples were diluted in E. coli rRNA.

RQ-PCR was set up with a master mix containing the tested forward ASO primer, consensus (germ-line) reverse primer, consensus (germ-line) probe and 10^{-2} dilution of the patient's diagnostic DNA (see below). Efficient primers, suitable for MRD detection with sensitivity $\geq 10^{-4}$, should give good amplification curves with Ct of the sample (10^{-2} dilution) < 30 and Ct of the polyclonal DNA > 10 cycles higher, ideally negative. The primer with the best parameters (lowest CT of the sample, biggest difference between Ct of the sample and the polyclonal DNA, good quality amplification curve) was chosen for the sensitivity and specificity testing and MRD detection.

The sensitivity and specificity of the RQ-PCR performed with the designed ASO primer were tested with the whole dilution series of the patient's diagnostic sample (in some cases it could be done at the same time with MRD detection). The reproducible sensitivity, enabling MRD quantification, and the absolute sensitivity threshold of the reaction were defined according to the studies of reference [Pongers-

Willemse *et al* 1998, van Dongen *et al* 2003, Verhagen *et al* 2000, Langerak *et al* 2001, van der Velden *et al* 2002b and c, Bruggemann *et al* 2004] (Table 4.11. and Protocol 28).

Table 4.11. Definition of specificity and sensitivity of RQ-PCR for IG/TCR rearrangements using ASO primer approach.

RQ-PCR for IG/TCR rearrangements – sensitivity/specificity definition	
reproducible sensitivity:	the last dilution of the patient's sample that comes up positive and where - its Ct is ≤ 40 - the difference in Ct of the reaction in duplicate is ≤ 1.5 cycles - the mean Ct of the sample is ≥ 3 cycles lower than the mean Ct of the polyclonal DNA - the standard curve has a slope between 3.3 – 3.7 and correlation coefficient ≥ 0.98
absolute sensitivity:	the last dilution of the patient's sample that comes up positive and where - at least one of the reactions carried out in duplicate comes up positive - at least one Ct of the reactions carried out in duplicate is ≥ 1 cycle lower than the lowest Ct of the polyclonal DNA

4.11.8. RQ-PCR

The RQ-PCR of clonal *IG/TCR* rearrangements was performed in similar way as RQ-PCR for chromosomal aberrations. The selected designed ASO forward primer and consensus reverse primer and probe, 6FAM-TAMRA labelled, were used [Pongers-Willemse *et al* 1998, Verhagen *et al* 2000, van der Velden *et al* 2002b and c, Bruggemann *et al* 2000 and 2004] (Table 4.12.). Together with the target clone-specific region, albumin as a control gene was always amplified to enable corrections for the starting amount and quality of DNA. A 10-fold dilution series (10^0 – 10^{-6}) of DNA from patient's sample (100 ng / µl) in human polyclonal DNA (100 ng / µl) was used for a relative quantification of the target and control genes.

The RQ-PCR master mix containing forward and reverse primers, probe, 2X TaqMan Universal Master Mix and sdH_2O to the final volume of the reaction of 25 µl was prepared. Template cDNA 5 µl (100 ng / µl) was added into the reaction. Reactions for detection of the clone-specific region in samples were carried out in triplicate, reactions for the control gene and for dilution series in duplicate. The RQ-PCR was performed on ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After preincubation and heat activation of AmpliTaq enzyme, 50 cycles were run.

Table 4.12. Consensus germ-line primers and probes for RQ-PCR of IG/TCR clonal rearrangements.

Consensus germline primers and probes for RQ-PCR of IG/TCR clonal rearrangements		
Gene	Code	Sequence 5' - 3'
IGH [Verhagen 2000]		
Rvs primers:	JH1-intron	CGC TAT CCC CAG ACA GCA GA
	JH2-intron	GGT GCC TGG ACA GAG AAG ACT
	JH3-intron	AGG CAG AAG GAA AGC CAT CTT AC
	JH4-intron	CAG AGT TAA AGC AGG AGA GAG GTT GT
	JH5-intron	AGA GAG GGG GTG GTG AGG ACT
	JH6-intron	GCA GAA AAC AAA GGC CCT AGA GT
Probes:	JH1.2.4.5	CCC TGG TCA CCG TCT CCT CAG GTG
	JH3	CAA GGG ACA ATG GTC ACC GTC TCT TCA
	JH6	CAC GGT CAC CGT CTC CTC AGG TAA GAA
IGK [van der Velden 2002c]		
Rvs primers:	KDE	TAC AGA CAG GTC CTC AGA GGT CAG
Probes:	KDE	AGC TGC ATT TTT GCC ATA TCC ACT ATT TGG AGT
TCRG[van der Velden 2002b]		
Rvs primers:	JG1.1/2.1	TAT ACT GAG GCC AGG AAT GTG ACA TA
	JG1.3/2.3	TTC CTG CCT TCC CTC TAT TAC CT
Probes:	JG1.1/2.1	ACC AGG TGA AGT TAC TAT GAG CTT AGT CCC TTC AG
	JG1.3/2.3	TGT CAC AGG TAA GTA TCG GAA GAA TAC AAC ATT TCC
TCRD [Langerak 2001]		
Rvs primers:	DD3	TTG CCC CTG CAG TTT TTG TAC
	JD1-cons1	TTA GAT GGA GGA TGC CTT AAC CTT A
	JD1-cons2	CCA TTT CCC ATT TCA GGA TTA
Probes:	DD3	ATA CGC ACA GTG CTA CAA AAC CTA CAG AGA CCT
	JD1	CCC GTG TGA CTG TGG AAC CAA GTA AGT AAC TC
TCRB [Bruggemann 2004]		
Rvs primers:	JB1.1	ACA CTG CCT AGA CGT TTT CTT G
	JB1.2	CTG GGG GTC GGA ATG GAT GTT
	JB1.3	CGA CAG GTC GGA ACT GAA TGA GT
	JB1.4	TGG AAT ACT ATG TGA TAG GGC TTT CTT
	JB1.5	GGG ACT AAG ACG GTT GAA TGG AT
	JB1.6	TCG GGG GTA TGG ACA GTG TCA
	JB2.1	GGG AAG AAT GGA TCG TGC CAC T
	JB2.2	GTT GGC GGA GGA ATG GGT
	JB2.3	GGG CGA ATG GCT CGT GAC A
	JB2.4	CCG CCG GGG TCG AAT G
	JB2.5	AGT GGC TCG TGG TCC TCG
	JB2.6	AGT GGG TCG TGC CAG TCG
	JB2.7	CCG ACC TTC CAC CCC TCT
Probes:	JB1.1	TTG GAC AAG GCA CCA GAC TCA CAG TTG
	JB1.2	CCT TCG GTT CGG GGA CCA GGT TAA
	JB1.3	TTG GAG AGG GAA GTT GGC TCA CTG TTG TAG
	JB1.4	TGG CAG TGG AAC CCA GCT CTC TGT C
	JB1.5	CAG CAT TTT GGT GAT GGG ACT CGA CTC TC
	JB1.6	CCA CTT TGG GAA TGG GAC CAG GCT
	JB2.1	ATG AGC AGT TCT TCG GGC CAG GGA
	JB2.2	TTG GAG AAG GCT CTA GGC TGA CCG TA
	JB2.3	AGT ATT TTG GCC CAG GCA CCC GG
	JB2.4	CGG GAC CCG GCT CTC AGT GCT
	JB2.5	CAG TAC TTC GGG CCA GGC ACG C
	JB2.6	AAC GTC CTG ACT TTC GGG GCC G
	JB2.7	CGG GCA CCA GGC TCA CGG TC
Albumin [Pongers-Willemse 1998]		
Fwd primer:	ALB-Fwd	TGA AAC ATA CGT TCC CAA AGA GTT T
Rvs primer:	ALB-Rvs	CTC TCC TTC TCA GAA AGT GTG CAT AT
Probe:	ALB	TGC TGA AAC ATT CAC CTT CCA TGC AGA

The results of RQ-PCR were analysed by ABI PRISM 7700 Sequence Detection System software and Microsoft Excel software. The threshold was set at 0.05, the base line was set between cycles 3 – 15. Based on the dilution series, standard curves for the clone-specific region and control gene were created and their slope, intercept and correlation coefficient evaluated. A sample was considered positive if at least one of the triplicate or duplicate RQ-PCR reactions was positive (above the defined sensitivity/specificity threshold). For each sample, mean Ct ± standard deviation and mean ΔCt ± standard deviation were calculated. The relative amount of the clone-specific region sequence was calculated from the standard curve method (Protocol 29).

The diagnostic and all the follow-up samples of each patient were analysed by RQ-PCR, preferably together on one TaqMan plate. For every patient, analysis of two or three independent *IG/TCR* targets was required to prevent false negative results due to clonal evolution or switch. The independent *IG/TCR* targets involved junctional regions of different types of rearrangements - eg. *IGH* and *IGK* etc., or *IGH* V1-JH and IGH V3-JH etc. in one patient (two different junctional regions of the same rearrangement - eg. V-D and D-J junctional regions of IGH V3-JH rearrangement - were not recognised as different independent targets). The MRD status and levels in the samples were defined according to relative amount of the clone-specific region detected (Table 4.13.).

Table 4.13. Definition of MRD presence and levels detected by PCR of clonal IG/TCR rearrangements.

MRD status and levels detected by PCR of clonal IG/TCR rearrangements:	
MRD negative	all reactions negative
MRD positive	at least one of the reactions positive
very high level	$\geq 10^{-1}$ of the amount at diagnosis (corrected to control gene)
high level	$10^{-1} - 10^{-2}$
medium level	$10^{-2} - 10^{-3}$
low level	$10^{-3} - 10^{-4}$
very low level	$< 10^{-4}$
MRD positivity > 10^{-4} is recognised as prognostically significant	

4.12. Detection of WT1 overexpression by RT-PCR and RQ-PCR

At present, there is no standardised RT-PCR or RQ-PCR assay for detection of *WT1* expression. We carried out several initial experiments according to the different methods published [Menssen *et al* 1995, Kreuzer *et al* 2001, Cilloni *et al* 2002]. As neither of them gave satisfying results in our hands and all of them use non-standard reaction conditions, we then decided to establish, optimise and validate RT-PCR and RQ-PCR for detection and quantification of *WT1* and its main isoforms according to the BIOMED-1 Concerted Action and Europe Against Cancer Program (EAC) recommendations and protocols,

respectively [van Dongen *et al* 1999, Gabert *et al* 2003, Beillard *et al* 2003]. These protocols are designed for detection of fusion gene transcripts in leukemia and are convenient for detection of gene overexpression.

4.12.1. RT reaction

The cDNA synthesis was performed starting from 1 μg of total RNA according to the EAC recommendations and in the same way like for PCR detection of chromosomal aberrations (Protocol 12).

4.12.2. RT-PCR for detection of WT1 and its main isoforms

First round and nested PCR for detection of *WT1* and its four main isoforms were designed according to the BIOMED-1 protocols. Primers for the first round PCR of *WT1* were adopted from Cilloni *et al* [Cilloni *et al* 2002], primers for the nested PCR of *WT1* and for the first round and nested PCR of *WT1* isoforms were designed using PrimerExpress 2.0 software and by manual examination of the sequences. Two sets of primers for each reaction were designed and tested and the one with better performance was selected (Table 4.14. and Figure 4.7.). $MgCl_2$ titration was carried out to optimise all the reactions; and $MgCl_2$ concentration of 3.0 – 3.5 mM was consequently chosen.

Table 4.14. Primers designed and selected for RT-PCR of WT1 and its isoforms.

PCR primers for detection of total WT1, first round and nested reactions		
Primer code	5' Position (size)	Sequence 5' - 3'
WT1-A (EX7)	1329 (21)	GGC ATC TGA GAC CAG TGA GAA
WT1-B (postEX10)	1811 (22)	GAG AGT CAG ACT TGA AAG CAG T
WT1-C (EX7)	1404 (22)	CCA CTT ACA GAT GCA CAG CAG G
WT1-D (EX10)	1699 (22)	TGT TGT GAT GGC GGA CTA ATT C
PCR primers for detection of WT1 isoforms, first round and nested reactions		
Primer code	5' Position (size)	Sequence 5' - 3'
WT1- EX4/6	1127 (20)	CCT TAA AGG GCC ACA GCA CA
WT1-EX5	1153 (21)	TCC AGC TCA GTG AAA TGG ACA
WT1-EX9/10	1630 (21)	AGG GCT TTT CAC CTG TAT GAG
WT1-KTS	1619 (22)	CTT GTT TTA CCT GTA TGA GTC C
WT1-EX6	1236 (21)	CGG AGC CCA ATA CAG AAT ACA
WT1-EX8	1478 (21)	CTT CGT TCA CAG TCC TTG AAG
All positions correspond to WT1 mRNA variant D sequence, NM_024426.2. Sequences representing exon 5 (1137-1187) and KTS (1610-1618) are excluded in case of primer WT1-EX4/6 and WT1-EX9/10, respectively.		

The PCR reaction was set up and performed in the same way as first round and nested PCR of chromosomal aberrations (Protocol 13 and 14). Results were considered positive if a clear band of the appropriate size was present.

RT-PCR for detection of total *WT1* was designed as a reference reaction for the following establishment of RT-PCR for *WT1* isoforms and RQ-PCR for *WT1*. It was tested on a number of different samples including cell lines (K562, BV173), normal peripheral blood, normal bone marrow and acute leukaemia bone marrow. As *WT1* expression could be detected in all of these samples, E. coli rRNA was used as a negative control. This method was not employed routinely for examination of patients' samples, however it could be applied for confirmation of results of other assays.

RT-PCR for detection of main *WT1* isoforms was tested on a limited number of samples (K562, normal peripheral blood and bone marrow, acute leukaemia bone marrow). It has not been used for routine examination of patients' samples.

4.12.3. RQ-PCR for detection of WT1 and its main isoforms

RQ-PCR for detection and quantification of *WT1* and its main isoforms - *WT1*-exon5 [+] and *WT1*-KTS [+] - were designed according to EAC protocols. Primers and a probes, 6FAM and TAMRA labelled, were designed using PrimerExpress 2.0 software; again, two sets of primers and probe were designed and tested and those giving better performance were chosen (Table 4.15. and Figure 4.7.). Control genes (CG) *ABL* or *B2M* were amplified together with *WT1*. *ABL* was detected using primers and probe as described in EAC protocols [Gabert *et al* 2003], *B2M* by Pre-developed TaqMan assay reagents control kit for human B2M (Applied Biosystems). A set of WT1 plasmid DNA calibrators in 10 log dilutions ($10^1 - 10^6$) was prepared (see below) and used for absolute quantification of *WT1*; plasmid DNA calibrators for control genes were purchased (Ipsogen).

The RQ-PCR reaction was set up and performed in the same way as RQ-PCR for detection of fusion genes (Protocol 16). Detection of *WT1* was carried out in triplicate reaction, detection of CG and calibrators in duplicate.

The results of RQ-PCR were analysed also in the same way as in case of RQ-PCR of fusion genes. For *WT1* and control genes the threshold was set at 0.1 to be in the exponential phase, and the baseline between 3 - 15, except for *B2M* with the baseline 3 - 10. The plasmid calibrator standard curves for *WT1* and control genes were created, and their slope, intercept and correlation coefficient evaluated. A sample was considered positive if at least one of the triplicate or duplicate RQ-PCR reactions was positive. For each sample, mean Ct ± standard deviation, mean ΔCt ± standard deviation, WT1 copy number (WT1 CN), control gene copy number (CGCN) and WT1 normalized copy number (WT1 NCN) were calculated. Validation analysis for applicability of comparative ΔΔCt method was performed (Bulletin 2, 10/2001, Applied Biosystems). The sensitivity levels of the individual experiments may be assessed according to the EAC recommendations.

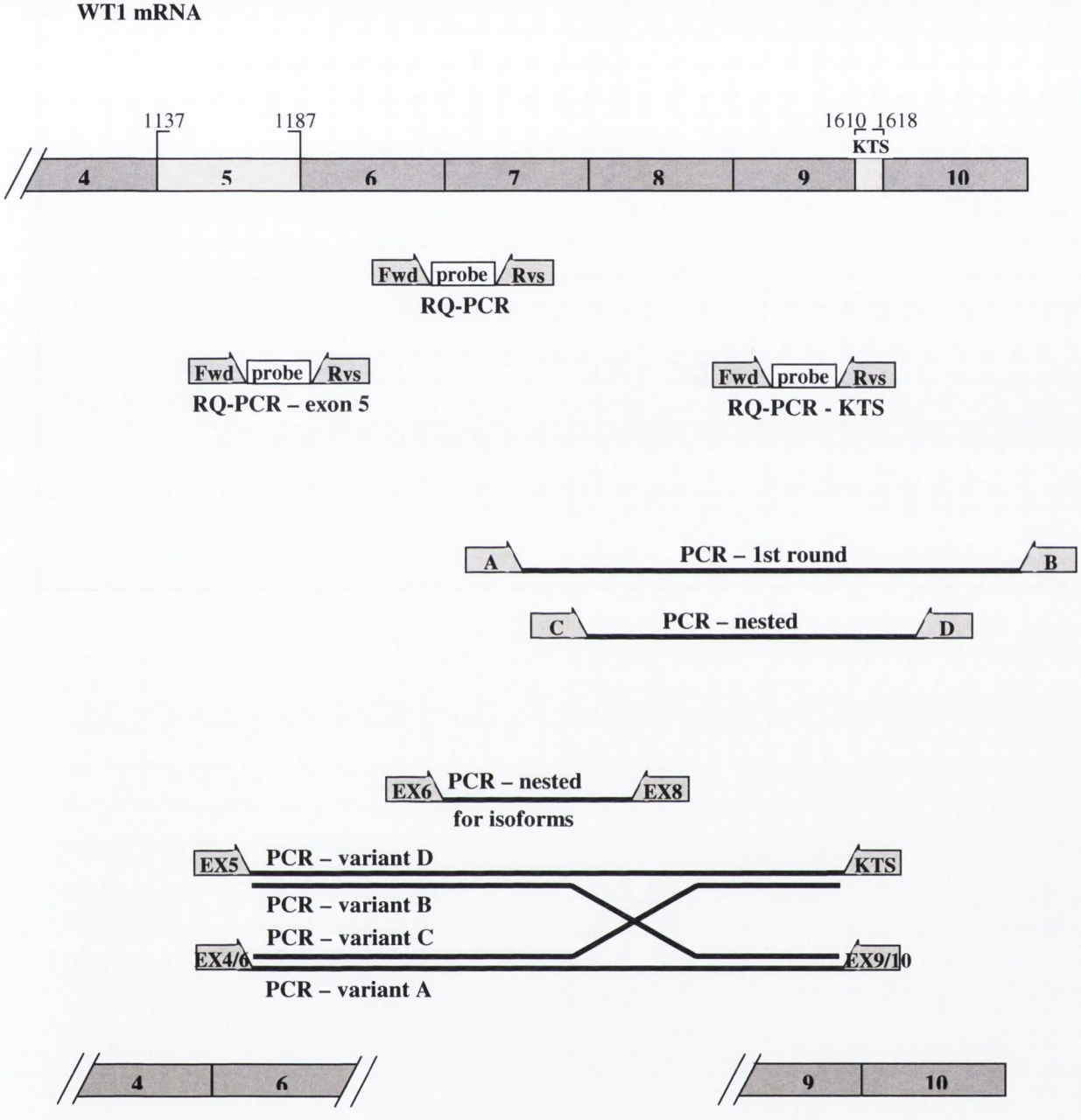


Figure 4.7. Scheme of the designed RT-PCR and RQ-PCR reactions for detection of *WT1* and its isoforms; positions of the primers and probe.

Table 4.15. Primers and probes designed and selected for RQ-PCR of WT1 and its main isoforms.

RQ-PCR primers and probes for detection of total WT1		
Primer/probe code	5' Position (size)	Sequence 5' - 3'
WT1-Fwd (EX6)	1253 (21)	TAC ACA CGC ACG GTG TCT TCA
WT1-Rvs (EX7)	1338 (21)	CTC AGA TGC CGA CCG TAC AAG
WT1-Probe (EX6/7)	1275 (25)	6FAM-AGG CAT TCA GGA TGT GCG ACG TGT G-TAMRA
RQ-PCR primers and probes for detection of WT1 main isoforms		
Primer/probe code	5' Position (size)	Sequence 5' - 3'
EX5-Fwd (EX5)	1154 (22)	CCAGCTCAGTGAAATGGACAGA
EX5-Rvs (EX6)	1230 (21)	GATGGGCGTTGTGTGGTTATC
EX5-Probe (EX5/6)	1180 (26)	6FAM-CAGAGCAACCACAGCACAGGGTACGA-TAMRA
KTS-Fwd (EX9)	1528 (26)	GTGAAACCATTCCAGTGTAAAACTTG
KTS-Rvs (EX9/KTS)	1618 (26)	TTGTTTTACCTGTATGAGTCCTGGTG
KTS-Probe (EX9)	1558 (24)	6FAM-CGAAAGTTCTCCCGGTCCGACCAC-TAMRA
All positions correspond to WT1 mRNA variant D sequence, NM_024426.2.		

The RQ-PCR reaction for detection and quantification of *WT1* expression was first validated on a number of different samples including K562 cell line, normal peripheral blood, normal bone marrow, peripheral stem cells, MRD negative regenerating bone marrow after chemotherapy, bone marrow and peripheral blood from acute leukaemia and bone marrow from other haematological malignancies. E. coli rRNA was used as a negative control. The median and maximal ranges of *WT1* expression in control samples (normal peripheral blood, normal and regenerating bone marrow) were determined to set the limits of normal and abnormal levels of *WT1* expression in haematopoietic tissues.

The detection and quantification of *WT1* expression by RQ-PCR was performed in diagnostic bone marrow samples of all patients. In those patients with high levels of *WT1* overexpression (≥ 2 log above the upper limit of normal *WT1* expression in bone marrow), *WT1* could be used as an MRD marker and its expression evaluated in all follow-up samples of these patients. The MRD status and levels were then defined according to the WT1 NCN detected (Table 4.16.).

4.12.4. Specificity and sensitivity tests

The specificity of the RT-PCR and RQ-PCR reactions was assessed from positive controls (K562 cell line, patient's and normal bone marrow) and negative control (E. coli rRNA) that were always included and analysed together with the tested samples.

The sensitivity of the RT-PCR and RQ-PCR reactions was evaluated in a 10-fold dilution series (10^{-1} - 10^{-6}) of K562 cell line or patient's RNA diluted into E. coli rRNA (Protocol 17). For RQ-PCR, the sensitivity was also determined from the plasmid calibrator series.

The median and maximal ranges of *WT1* expression in normal haematopoietic tissues defined the sensitivity threshold for detection of abnormal *WT1* overexpression and MRD monitoring by *WT1*.

Table 4.16. Definition of MRD presence and levels detected by PCR of WT1 overexpression.

MRD status and levels detected by PCR of WT1:	
MRD negative	WT1 NCN within the normal limits of expression in bone marrow
MRD positive	WT1 NCN above the normal limits of expression in bone marrow
high level	WT1 NCN (WT1 CN / CGCN) ≥ 2 log
medium level	1 – 2 log
low level	< 1 log above the normal limits
MRD positivity above the normal limits is recognised as prognostically significant	

4.13. Preparation of WT1 calibrators for real-time PCR quantification

WT1 calibrators for RQ-PCR absolute quantification of *WT1* expression were prepared according to the EAC recommendations [Gabert *et al* 2003].

4.13.1. Cloning of a PCR product

Cloning of a part of *WT1* gene containing the full sequence detected by RQ-PCR was carried out using TOPO TA Cloning Kit (Invitrogen). The method of TOPO TA cloning provides fast and efficient way of direct insertion of Taq DNA polymerase-amplified PCR products into a plasmid vector. The plasmid vector supplied in the kit (pCR 2.1-TOPO) is linearised with single 3'-thymidine overhangs and topoisomerase I covalently bound to the ends of the vector. Topoisomerase I cleaves the phosphodiester backbone of one DNA strand after 5'-CCCTT sequence. The energy released from this reaction is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue of topoisomerase I. At presence of 5' hydroxyl of the original cleaved strand (or other DNA sequence complimentary at the cleaved sites) this reaction can be reversed with re-joining the DNA strands and releasing topoisomerase I. The map of the pCR 2.1-TOPO vector is included in the TOPO TA Cloning guidebook (version Q, July 2003, Invitrogen).

First, RT-PCR reaction with primers WT1-EX5 + WT1-KTS was performed to amplify the target *WT1* sequence, according the standard protocol. At the end of the reaction, an extension step was included, allowing an addition of a single deoxyadenosine to the 3' ends of the PCR product (by nontemplate-dependent terminal transferase activity of Taq DNA polymerase). The efficiency of the PCR reaction was checked by electrophoresis of the product on 2 % agarose gel, the concentration of PCR product was

estimated by comparison with a mass ladder, and an optimal amount of PCR product per one cloning reaction was then calculated.

The cloning reaction was performed by incubation of the PCR product with the vector in a salt solution at room temperature.

After that, the vector with the cloned gene insert was immediately transformed into chemically competent TOP10 E. coli strain (method of chemical transformation). After incubation of the vector with the cells on ice, the cells were heat-shocked (at 42 °C) and recovered by addition of SOC medium and incubation at 37 °C. The transformed cells and their 10-fold dilution were then spread on pre-warmed selective LB plates (with Ampicillin and X-gal) and incubated for about 12 hours (overnight).

Cells transformed with a linearised vector alone, a control circular vector (pUC19), and with no vector (grown on LB plates with and without Ampicillin) were used as controls.

The positive clones were then identified as white or light blue colonies (the pCR 2.1-TOPO vector contains Ampicillin-resistance gene, and lacZ gene that is disrupted in the linearised vector or a vector with the cloned gene insert; thus colonies that can grow on LB agar with Ampicillin and do not metabolise X-gal, leading to blue colour of the cells, may be considered to carry the plasmid with the cloned sequence). The transformants were then screened for the presence of the right plasmid by PCR reaction (the same PCR reaction with primers WT1-EX5 + WT1-KTS that has been used to generate the *WT1* sequence for cloning) (Figure 4.8.) and consequently sequenced to verify the correct sequence of the clones (using M13 Fwd and M13 Rvs primers from the TOPO TA Cloning kit).

Following this, a frozen stock of transformants was prepared, and remaining bacteria were grown for bulk production of the plasmid (Protocol 30).

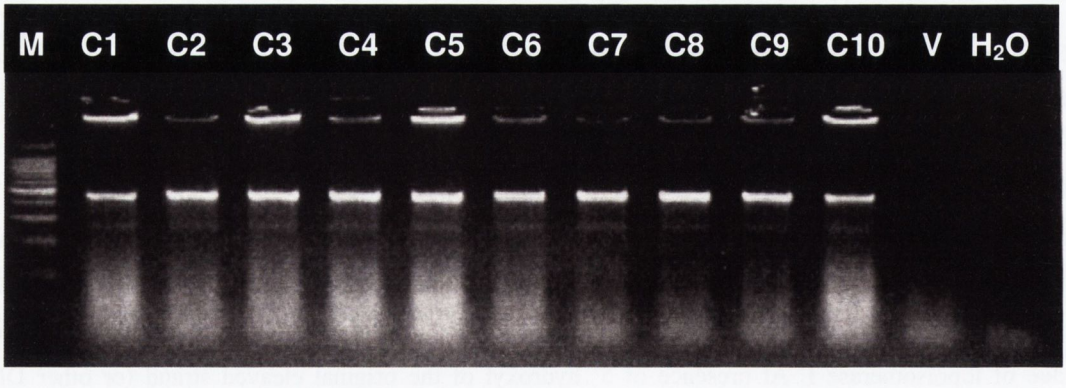


Figure 4.8. Screening transformants for the presence of vector containing WT1 insert (C - screened clones, all of them containing the WT1 cloned sequence, V - control clone transformed with an empty vector only).

4.13.2. Production and extraction of plasmid DNA

The bacterial clones containing the correct plasmid were cultured in LB medium with Ampicillin by standard techniques (Protocol 18). After a sufficient amount of the culture was obtained, the cells were separated by centrifugation and lysed, following plasmid extraction.

The plasmid DNA was extracted from the cells using Qiagen HiSpeed Plasmid Midi Kit. The extraction and purification method is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low molecular weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer, concentrated and desalted by isopropanol precipitation and finally eluted by TE buffer or water.

Plasmid DNA extraction was performed according to the HiSpeed Plasmid Purification Handbook (Qiagen, December 2001). The quality of the extracted plasmid DNA was checked by electrophoresis on 1 % agarose gel. A part of the amount of the circular DNA plasmid was kept and stored at – 20 °C or – 80 °C, the rest was used for preparation of the plasmid calibrators (Protocol 31).

4.13.3. Linearising of plasmid DNA and preparing a stock of calibrators

The plasmid DNA was quantified by SPEC and the plasmid/copy number per 1 µl of DNA was calculated. The circular plasmid was consequently linearised by restriction enzyme digestion using EcoRI (the pCR 2.1-TOPO cloning vector contains EcoRI restriction sites close to the cloned gene insertion) and the product was run on 1 % agarose gel to confirm the efficiency of the digestion (Figure 4.9.).

The linearised plasmid was serially diluted in a solution containing Tris/EDTA and E. coli rRNA to get a set of WT1 plasmid DNA calibrators containing 10^1 – 10^6 WT1 copies / 5 µl and stored at – 20 °C (Protocol 32).

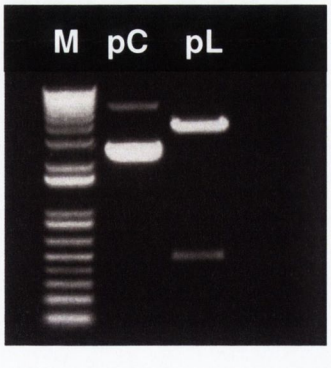


Figure 4.9. Checking the purity of extracted plasmid and efficiency of its restriction digestion. pC – extracted circular plasmid (upper weak band corresponding to a supercoiled form), pL – linearised plasmid (stronger band of about 3 900 bp corresponding to the original vector, and weaker band of about 500 bp to the cloned WT1 sequence extended at the ends of short vector sequences).

4.14. Detection of WT1 by flow cytometry and immunofluorescence microscopy

WT1 protein can be detected in cells expressing WT1 by monoclonal antibodies. Presence of WT1 protein in leukaemic blasts has been demonstrated by indirect immunofluorescence [Menssen *et al* 1995 and 1997]. Untill the present, there has been no assay for detection of WT1 protein by flow cytometry (FC) established or reported. Very preliminary work of colleagues from our lab found that anti-WT1 monoclonal antibodies could be potentially used for staining and analysing WT1 positive cells by FC (Mireille Crampe, Cancer Molecular Diagnostics, St. James's Hospital, personal communication). As FC offers a rapid, relatively simple, yet well reproducible and sensitive method of detection of intracellular antigens, which monoclonal antibodies are available against, we were interested in developing a method of WT1 detection by FC. Such an assay would have further advantages, like the possibility of correlating WT1 expression with expression of other leukaemia-associated antigens, to discriminate between vital and dying/death WT1 positive cells, or to analyse WT1 expression in defined subpopulations of cells (different lineages, stem cells, etc.).

Our previous experience with flow cytometric detection of intracellular antigens and FC MRD studies, and available literature on the methods of detection of intracellular antigens [Ormerod *et al* 2000, Millard *et al* 1998, Zamai *et al* 2002; DAKO, Caltag, Serotec and Becton Dickinson catalogues, information brochures and sheets] were the basis for this research.

4.14.1. Detection of WT1 protein by flow cytometry – indirect intracellular staining

Initial tests of WT1 detection by FC were done with primary (unlabelled) anti-WT1 mouse monoclonal antibody, clone 6F-H2 (DAKO), on several leukaemic cell lines (BV173, MV4-11) and acute leukaemia (ALL, AML) bone marrows as a positive and peripheral blood as a negative control using indirect intracellular staining method. As secondary antibodies, FITC- or PE-labelled goat anti-mouse antibodies (both DAKO) were used. The amounts of anti-WT1 and secondary antibodies were optimised by titration.

The indirect intracellular staining involved fixation and permeabilisation of the cells (using IntraStain kit, DAKO), incubation with primary anti-WT1 antibody (unlabelled mouse anti-human), washing and blocking, incubation with secondary antibody (FITC- or PE-goat anti-mouse), further washing and blocking steps including incubation with normal mouse serum, and then eventually direct staining for other surface or intracellular antigens followed by final washing and fixation (Protocol 33). The sample was acquired and analysed on flow cytometer in a standard way.

4.14.2. Detection of WT1 protein by flow cytometry – direct intracellular staining

After the initial tests, a method of direct intracellular staining for WT1 was set up to reduce unspecific binding and improve the possibility of simultaneous staining of WT1 together with other antigens. FITC-labelled anti-WT1 antibody, clone 6F-H2 (DAKO primary antibody, labelled by DAKO for us as a customised product, mouse Ig concentration 300 mg / l, molar fluorochrome / protein ratio 5.7) was used

for this purpose and titrated to chose the optimal amount (5 μl per 10^6 cells). The staining was performed on K562 cell line and ALL bone marrow samples as a positive control, and peripheral blood and normal bone marrow samples as a negative control.

The staining procedure was carried out according the protocol for direct intracellular staining using IntraStain kit (DAKO) (Protocol 10), and samples were acquired and analysed as usual.

To confirm there was no expression of WT1 protein on cells surface, staining for WT1 according to the protocol of FC staining for surface antigens (Protocol 9) was once performed.

4.14.3. Detection of WT1 protein by flow cytometry – optimisation of the method

In order to increase the discrimination between the positive and negative samples and improve the method sensitivity and specificity, the key issues of the flow cytometric detection of WT1 were optimised. These included optimisation of fixation/permeabilisation procedure and washing/blocking steps, and the choice of an antibody and fluorochrome with the best performance.

As the fixation and permeabilisation processes may considerably influence the efficiency of staining for an intracellular antigen as well as the degree of unspecific antibody binding within the cell, a special effort was made to find the most suitable method of WT1 staining. Following fixation and/or permeabilisation methods were consequently tested: commercial kits for intracellular staining – apart from IntraStain (DAKO) also Fix and Perm (Caltag) and FACS Lysing and FACS Permeabilising 2 solutions (BD); IntraStain and Fix and Perm kits with cold methanol step included between fixation and permeabilisation; paraformaldehyde followed by methanol, paraformaldehyde followed by saponine, methanol alone, saponine alone; and various incubation times and temperatures.

To decrease possible unspecific antibody binding, more washing and several different blocking procedures were tested. Apart from standard washing with CellWash/FBS, washing in PBS/BSA/SA and pre-incubation with normal mouse serum, normal goat serum, anti-Fc antibodies and unlabelled isotype control (mouse IgG1) were tested.

The anti-WT1 antibodies that have become available during development of this method included rabbit polyclonal antibody (raised against a synthetic peptide derived from the C-terminal sequence of huWT1); and mouse monoclonal antibodies (IgG1) clone 6F-H2 (against truncated huWT1 corresponding to N-terminal amino acids 1 – 181), clone MWT6, MWT12 and WLM04 (all against rhuWT1 corresponding to amino acids 1 – 318). All these antibodies are designed for immunohistology, some of them also for immunofluorescence or Western blotting applications. No anti-WT1 antibody designed for flow cytometry application was available. After considering the characteristics of the different antibodies and information about their performance requested from the supplying companies, monoclonal mouse anti-WT1 clone WLM04 (NeoMarkers) was chosen for the final tests.

Apart from FITC, other fluorochromes giving brighter signals were investigated. They included particularly PE and a new generation of fluorochromes (AlexaFluor). After comparison of their properties, labelling procedures and costs, AlexaFluor488 was selected for labelling of the primary anti-WT1antibody. This fluorochrome is spectrally similar to FITC but much brighter and more photostable.

The anti-WT1 clone WLM04 antibody (NeoMarkers) was labelled with AlexaFluor488 using AlexaFluor488 Monoclonal Antibody Labeling kit (Molecular Probes), according to the manufacturer's protocol, and the efficiency of labelling was confirmed by calculating the molar F/P ratio. The labelling process is based on a reaction of AlexaFluor488 succinimidyl ester moiety with primary amines of proteins to form stable dye-protein conjugates. This labelling method was preferred to a newer labelling technology (zenon – fluorochrome-labelled Fab fragments that bind to the Fc portion of the monoclonal antibody) because of minimal risk of unspecific background and minimal increase of the antibody size, important in case of intracellular staining.

The anti-WT1-AlexaFluor488 antibody was diluted in PSA/BSA/SA to working concentration of 300 mg [mouse Ig] / l and its optimal amount for staining was determined by titration (5 µl per 10^6 cells). The finalised protocol for detection of WT1 protein by FC was then proposed, employing this antibody and intracellular staining with Fix and Perm (Caltag) kit (Protocol 34).

4.14.4. Detection of WT1 protein by immunofluorescence microscopy

Detection of WT1 by immunofluorescence microscopy was performed as a reference method for the flow cytometry experiments.

First stainings were performed according to the published technique [Menssen *et al* 1997] using primary unlabelled anti-WT1 mouse monoclonal antibody (DAKO) and FITC-labelled secondary goat anti-mouse antibody (DAKO), with appropriate isotype control.

Later, directly labelled FITC-anti-WT1 (DAKO) and AlexaFluor488-anti-WT1 (NeoMarkers/Molecular Probes) mouse monoclonal antibodies were also used and the staining protocol was optimised for better results. This included testing different fixation (methanol ice-cold or –20°C, aceton, aceton+methanol), permeabilisation (methanol alone, Triton X-100, methanol and Triton X-100) and washing/blocking (PBS/BSA, PBS/Tween-20, normal goat serum) techniques, several antibody dilutions and different times and temperatures of incubation. The tests were carried out on K562 cell line, mononuclear cells from peripheral blood, normal bone marrow and bone marrow samples from acute leukaemia.

In the optimised protocol, the cells were centrifuged by cytospin onto glass slides, fixed in ice-cold methanol, weakly permeabilised with Triton X-100 and after washing/blocking the cells were incubated with the fluorochrome-labelled anti-WT1 antibody. Following another washing, the slides were mounted beneath cover glasses with fluorescence-preserving mounting medium and examined under a fluorescent microscope (Protocol 35).

In addition to the classic immunofluorescent staining, a part of the cell suspension labelled for flow cytometric detection of WT1 was centrifuged onto slides and examined under fluorescent microscope in several cases.

4.15. Statistical analysis of data

The initial analysis of the results of the different methods was performed either by qualitative evaluation (in RT-PCR) or by analysis with appropriate software – CellQuest, FlowJo (FC), ABI PRISM 7700 Sequence Detection System (RQ-PCR). The final analysis of the results together with clinical data was carried out using statistical software of Microsoft Excel, SPSS (version 11.0), InStat 3 and Prism 4 (both GraphPad).

The comparison of the different methods of MRD detection and correlation of their results was tested by Bland-Altman method and Spearman rank test. To quantitatively correlate MRD levels detected by different methods, the amount of MRD in follow-up samples was expressed as percentage of leukaemic cells expressing the specific MRD marker at diagnosis (Table 4.17.).

Comparisons of *WT1* expression in different subgroups of patients were performed by Mann-Whitney non-parametric U test and Kruskal-Wallis non-parametric test with Dunn's multiple comparisons post-test. The relation between *WT1* expression and patients survival was assessed from Kaplan-Meier survival curves and by log rank test. Spearman rank test was used to calculate correlation of *WT1* and CD34 expression. Univariate and multivariate analysis were performed using Cox proportional hazards model with WT1 data categorized into 3 groups and with data transformed ($\log_2$ transformation of *WT1* expression values and the absolute value of their subtraction from the mean).

The statistical evaluation of the data was done with a help of Tony Fitzgerald from Haughton Institute, St. James's Hospital, Dublin, whom I thank for some theroretical remarks, and Marek Omelka from Deptartment of probability and mathematical statistics, Faculty of mathematics and physics of Charles University, Prague, who kindly help with Cox regression analysis.

Table 4.17. Definition of MRD presence and levels detected by different methods.

MRD status and levels detected by FC or PCR:	
MRD negative	all MRD targets negative
MRD positive	at least one of the MRD targets positive
very high level	≥ 10 % of the amount at diagnosis
high level	1 - 9.99 %
medium level	0.1 - 0.99 %
low level	0.01 - 0.099 %
very low level	< 0.01 %
MRD positivity > 10^{-4} is recognised as prognostically significant	

5. Results

Optimisation of laboratory techniques

5.1. Flow cytometry detection of leukaemia-associated phenotypes (LAPs)

5.1.1. Overall characteristics

The method set up and optimisation have been shown to influence significantly the quality of the immunophenotyping results obtained.

Careful sample handling and its immediate initial processing is necessary for the correct quantification of an antigen and LAPs evaluation. FC detection of the most common antigens and LAPs was performed on a group of fresh samples, both normal and acute leukaemia bone marrows, and on the same samples kept in RPMI at 4 °C for 24 hour – 5 days. Even after 24 hours, there were major changes in the antigen expression observed, with loss of the antigen expression and increase in unspecific background. Within a number of days, the decrease of specific fluorescence could be as high as 50 % when compared to the original measurement (Figure 5.1.).

The optimised protocols for staining of surface and intracellular antigens gave very high level of discrimination of cellular populations and antigen expression. Even very small changes in incubation times and temperatures (e.g. changing room temperature in the lab over the day or over the seasons) could cause a noticeable decrease in quality of the results and an increase of unspecific background.

Individual compensation parameters were set for samples from each patient to correct for the individual differences in cell autofluorescence. This improved the separation of the cellular populations detected by FC, and most importantly reduced the risk of false positive and false negative results (Figure 5.2.)

The multiple gating strategy improved and simplified the detection of LAPs and reduced unspecific background, thus decreasing the risk of false positivity.

A standardised technique of sample preparation, acquisition and analysis was essential for comparison of results between samples taken from an individual patient at different time points or between patients' and normal control samples.

5.1.2. Applicability

The method was able to identify LAPs suitable for MRD monitoring in all acute childhood leukaemia samples examined. In most cases, more than 1 LAP could be identified and used for MRD detection.

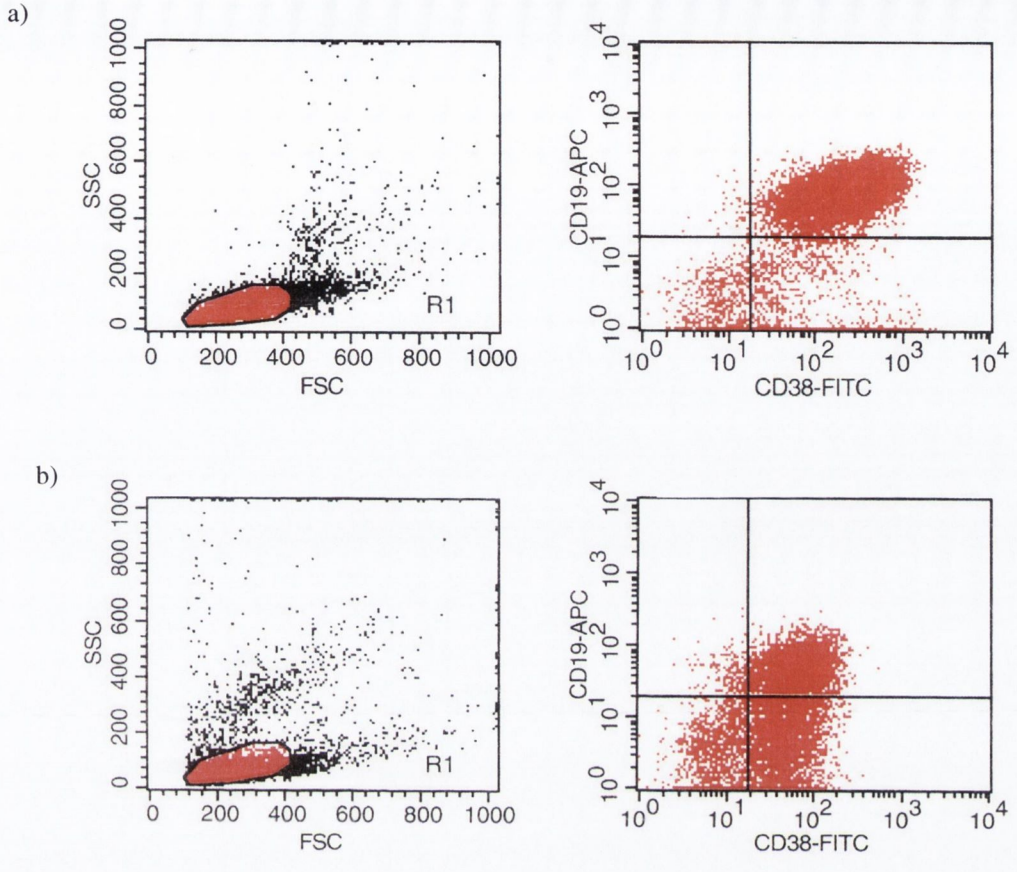


Figure 5.1. Changes in the expression of antigen and LAPs in bone marrow samples during the time after their collection; a) a fresh sample (4 hours after collection), b) the same sample stored (5 days after collection). A significant decrease in both CD19 and CD38 antigens expression and fluorescence intensity, and increase of unspecific background in the stored sample was observed.

5.1.3. Specificity

All LAPs used for MRD detection and monitoring fulfilled the required criteria for their specificity, with either zero or very low positivity (< 0.1 – 0.01 % positive of total cells) detected in control samples. The specificity of a particular LAP depended on its character (qualitative vs. quantitative, asynchronous vs. cross-lineage expression, etc.) and on the character of the detecting antibody (IgG1 with low unspecific binding vs. IgG2b or IgM with higher unspecific binding).

LAPs with high specificity (none or < 0.01 % positivity in control samples) were preferred whenever possible (Figure 5.3.).

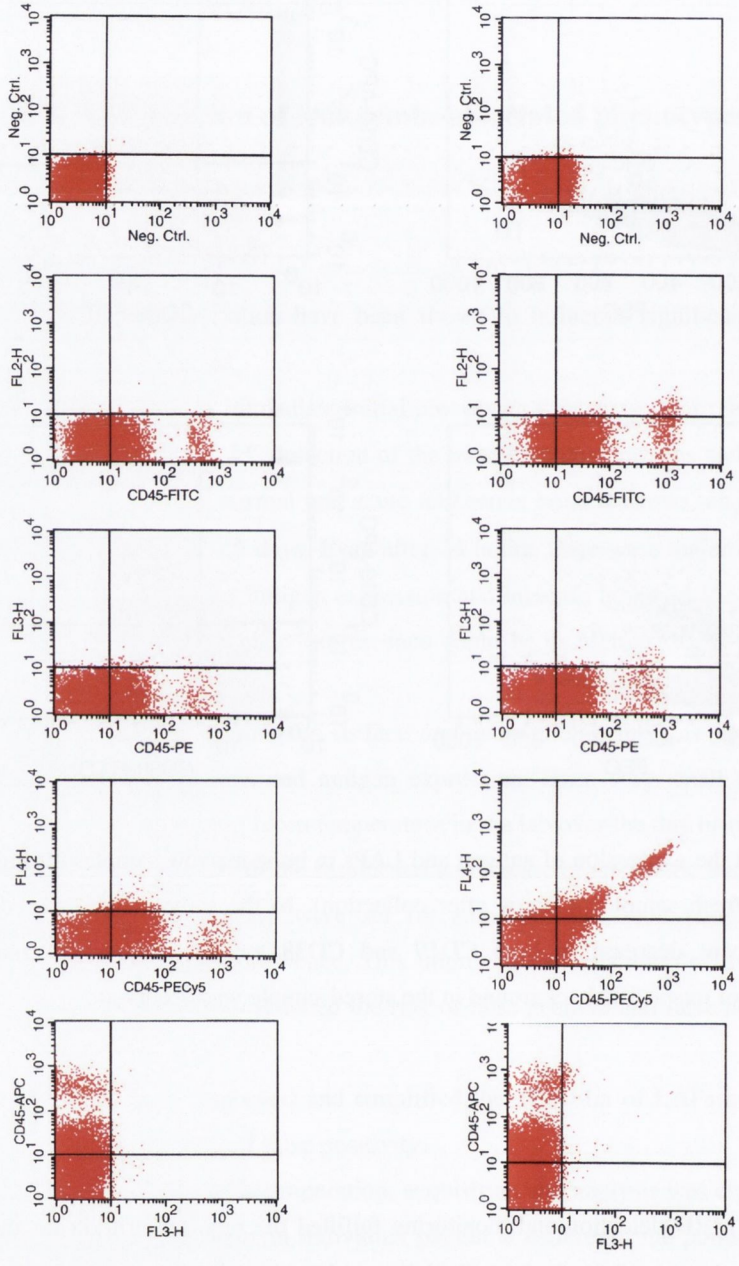


Figure 5.2. Comparison of individual compensation parameters settings (left) and use of common parameters settings (right) for the same patient. A spectral overlap is present when using the common compensation parameters (all fluorochromes detected also by channels other than the designated one, which is represented on the plots by dots overlapping into the upper right quadrant).

5.1.4. Sensitivity

The detected sensitivity of the method was found to be quite high and directly related to the number of acquired cells. As more than one positive event was required to consider a result to be positive, the sensitivity of the method was approximately 1 log lower than the number of acquired cells, 10^{-5} in case of 10^6 cells acquired, 10^{-4} in case of 10^5 cells acquired etc.

For the particular LAPs, their sensitivity threshold for MRD detection was determined by their background expression in control samples, that means by their specificity.

The sensitivity of MRD detection using different LAPs thus reached 10^{-3} – 10^{-5}, and was generally higher in ALL (10^{-4} – 10^{-5}; e.g. for LAPs CD58/10/34/19, CD66/10/34/19) than in AML (10^{-3} – 10^{-4}; e.g. for LAP CD7/13/45/117) (Figure 5.4.).

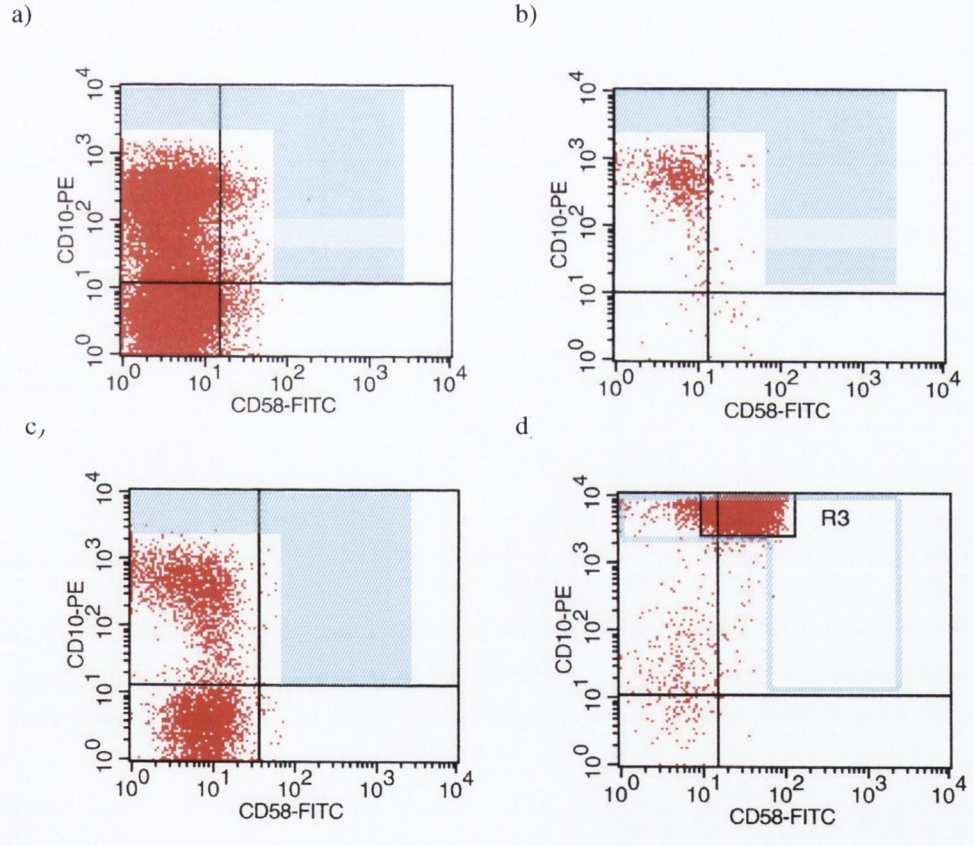


Figure 5.3. Specificity of LAPs used for FC MRD detection. Normal bone marrow (a) and regenerating bone marrow (b and c) with zero or very low (< 0.01 – 0.001 %) expression of LAP CD58/10/19 in the defined regions (in blue). B-ALL with abnormal expression of LAP CD58/10/19 in the vast majority of the leukaemic cell population (d).

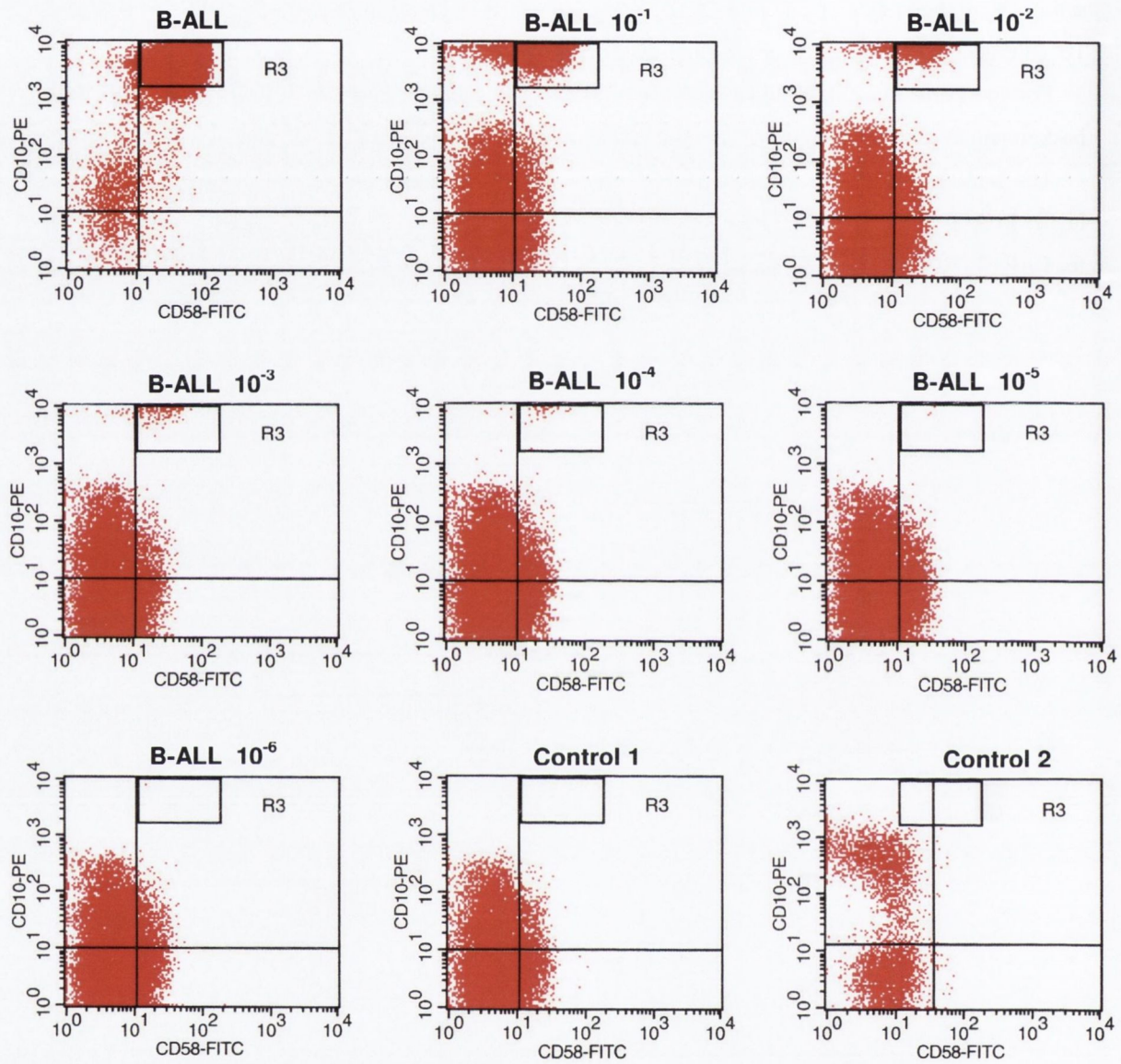


Figure 5.4. Sensitivity of FC MRD detection. B-ALL sample with LAP CD58/10/19 was diluted in 10-fold steps into a normal sample; two different normal samples were employed as controls. In this case, the achieved sensitivity reached 10^{-5}.

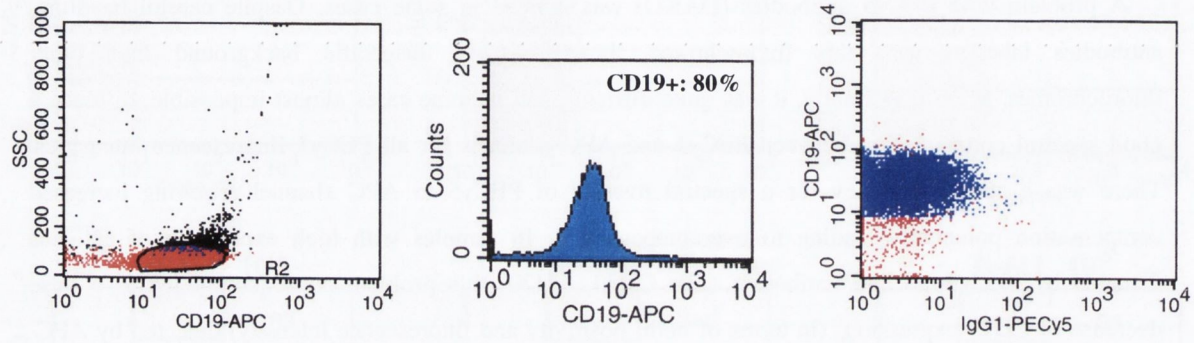
5.1.5. Reproducibility

The optimised method gave quite consistent results in comparison between experiments performed at different times.

5.1.6. Other factors potentially influencing immunophenotyping results

A problem with PECy5 antibodies (DAKO) was noticed in some cases. Despite careful handling, antibodies labelled with this fluorochrome showed higher unspecific background than other fluorochromes. More importantly, it was quite difficult, and in some cases almost impossible, to reach a good spectral compensation between PECy5 and APC channels for all PECy5 fluorescence intensities. There was a strong tendency for a spectral overlap of PECy5 to APC channel requiring increased compensation potentially leading to overcompensation. In samples with high expression of antigens detected by PECy5-labelled antibodies (like CD34, CD20) this problem consequently led to a false decrease of CD19 expression (in terms of both, positivity and fluorescence intensity) detected by APC-labelled antibody (Figure 5.5.). As CD19 was used for gating purpose and only CD19 positive cells were further analysed, the detected expression of all the other antigens in the combination decreased correspondingly. To solve this problem, several samples had to be re-analysed with antibody combinations excluding PECy5 label or a mathematical correction of the primary data was used (recalculating the number of positive cells within CD19 gate to the CD19 expression in the relevant isotype control, in diagnostic samples only).

IgG1/IgG1/IgG1/CD19



CD58/CD10/CD34/CD19

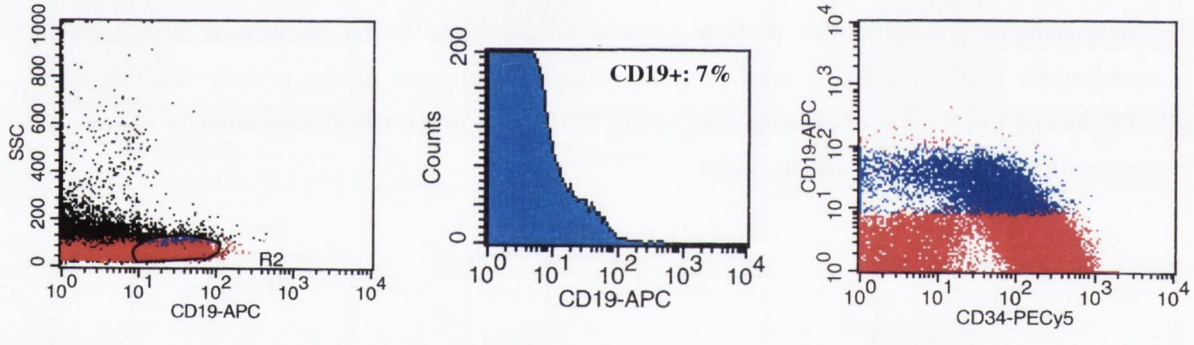


Figure 5.5. Decrease of detected CD19-APC labelled cells in B-ALL diagnostic sample with high CD34-PECy5 positivity. In an isotype control (above) the total number of detected CD19 positive cells is approximately 80 % of all cells. In a combination CD58-FITC/CD10-PE/CD34-PECy5/CD19-APC (below), there is a strong CD34 positivity while the total number of detected CD19 positive cells decreases to 7 % of all cells (more than 90 % difference!).

5.2. RT-PCR and RQ-PCR of chromosomal aberrations

5.2.1. Overall characteristics

The quality of the optimised RT-PCR for detection of chromosomal aberrations and the characteristics (robustness, standard curve slope, intercept and correlation coefficient, sensitivity threshold) of RQ-PCR for quantification of fusion gene transcripts were within the parameters required for MRD detection in the relevant protocols (BIOMED-1, EAC). Both RT-PCR and RQ-PCR produced reliable, reproducible, specific and sensitive results.

5.2.2. Specificity

Both RT-PCR and RQ-PCR were confirmed to be the most specific methods of MRD detection. After optimisation, neither false positive nor false negative results were observed in any of the experiments. Positive controls (cell lines, or patients' samples expressing the particular aberration) always gave a clear signal while negative controls (cell lines or patients' samples without the particular aberration, normal peripheral blood or bone marrow samples) clearly exhibited negative results (Figure 5.6., 5.7., also 5.23. and 5.26.).

5.2.3. Sensitivity

Sensitivity of first round PCR, nested PCR and RQ-PCR was generally very high and corresponding with the ranges expected. In positive cell line 10-fold dilution series, the reproducible sensitivity of first round PCR was 10^{-2} – 10^{-5}, nested PCR 10^{-4} – 10^{-6}, and RQ-PCR 10^{-4} – 10^{-6}. (Table 5.1., Figure 5.6. and 5.7.)

5.2.4. Reproducibility

The optimised assays gave quite consistent and comparable results between experiments for all chromosomal aberrations.

Table 5.1. Reproducible sensitivity of RT-PCR and RQ-PCR of chromosomal aberrations.

Reproducible sensitivity of RT-PCR and RQ-PCR of chromosomal aberrations (log sensitivity of positive cell line RNA 10-fold dilution series in negative cell line RNA)				
Chromosomal aberration		RT-PCR first round	RT-PCR nested	RQ-PCR
t(9;22)	BCR-ABL p190	-3	-4	-4
t(9;22)	BCR-ABL p210	-5	-5	-6
t(1;19)	E2A-PBX1	-4	-5	-6
t(4;11)	MLL-AF4	-4	-5	-5
t(12;21)	TEL-AML1	-3	-4	-5
microdel(1p)	SIL-TAL1	-2	-4	-4
t(8;21)	AML1-ETO	-4	-5	-6
t(15;17) (bcr1)	PML-RARA	-2	-4	-5
inv(16p) (type A)	CBFB-MYH11	-3	-6	-6

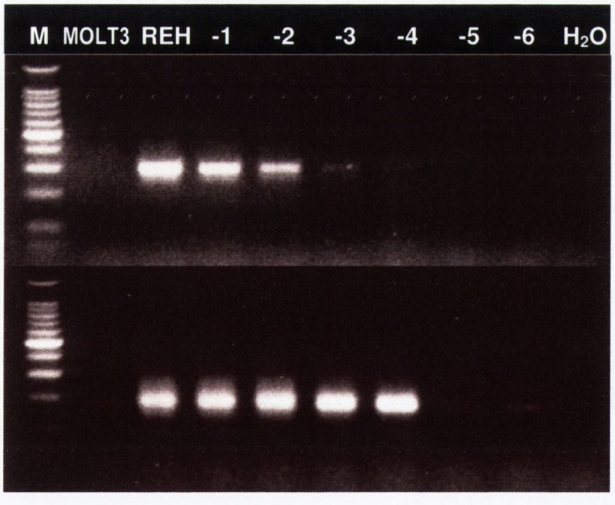


Figure 5.6. RT-PCR for the TEL-AML1 chromosomal translocation, first round PCR (above) and nested PCR (below); the sensitivity test was performed with the REH cell line dilution series (10^0 – 10^{-6}) and negative controls (MOLT3 cell line, H_2O).

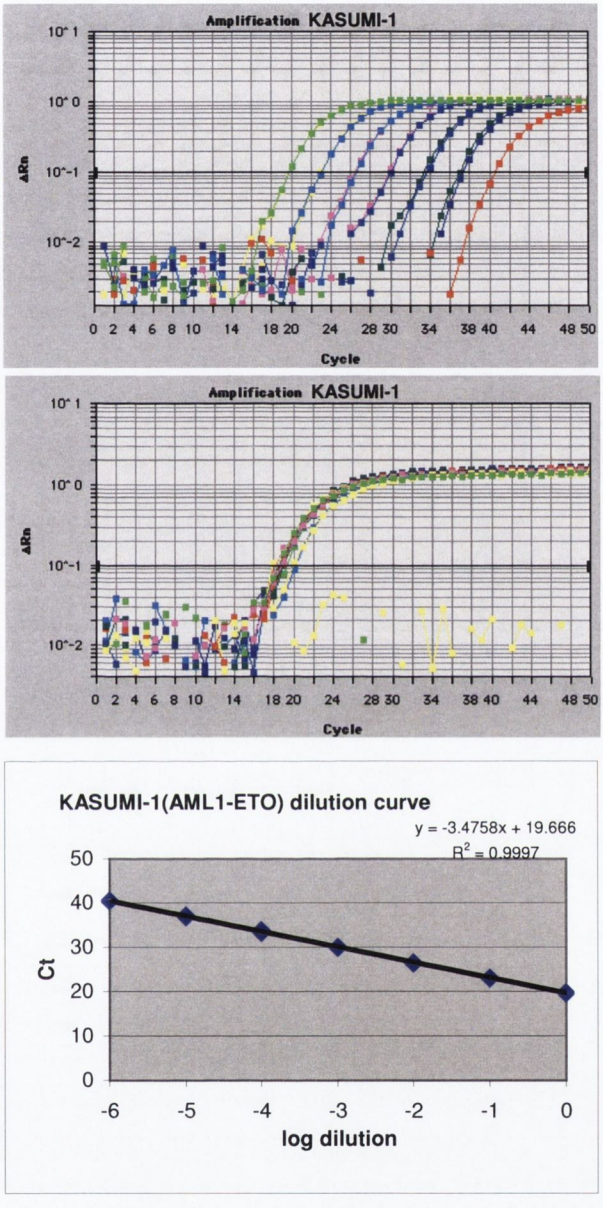


Figure 5.7. RQ-PCR for the AML1-ETO chromosomal translocation (top panel) and a control gene B2M (middle panel); sensitivity test with KASUMI-1 cell line dilution series (10^{0} – 10^{-6}) and negative controls (MOLT3 cell line, H_2O). Standard curve for KASUMI-1 dilution series with parameters indicated (lower panel).

5.3. PCR and RQ-PCR detection of clonal IG/TCR rearrangements

5.3.1. Overall characteristics

The identification of clonal *IG/TCR* markers and their use for MRD detection involves a series of steps - PCR screening reactions, product detection by gel or capillary electrophoresis, purification of PCR products, their sequencing, purification of sequencing products and their electrophoresis, analysis of the sequences and identification of V(D)J segments and junctional regions, design of ASO primers and their testing, and finally RQ-PCR MRD detection. After optimisation of all these steps, the applicability and quality of the method achieved the levels suggested by BIOMED-2 group and the reference studies [Pongers-Willemse *et al* 1998, van Dongen *et al* 2003, Verhagen *et al* 2000, Langerak *et al* 2001, van der Velden *et al* 2002b and c, Bruggemann *et al* 2004] and produced reliable and reproducible results.

5.3.2. Specificity

The patients' diagnostic samples and normal human polyclonal DNA were always included as positive or negative controls, respectively. The specificity of the method is indicated by the separation of clear positivity of the patient's diagnostic sample when compared to polyclonal DNA control background. In some cases, no background positivity was detected. In the remaining patients, low background positivity was present; here, the sensitivity threshold was set above this background to avoid problems in interpretation of the results (Table 4.11.). Hence, within the defined range of sensitivity, no false positive or false negative results were observed (Figure 5.8.).

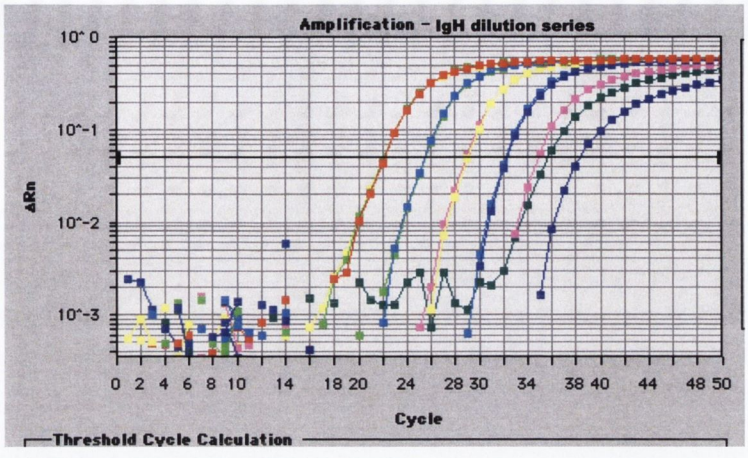
5.3.3. Sensitivity

The reproducible and absolute sensitivity of RQ-PCR detection of clonal *IG/TCR* rearrangements were defined according to the adopted criteria (Table 4.11.). The minimum required sensitivity of 10^{-4} was reached in all cases; the vast majority of patients achieved reproducible sensitivity of 10^{-4} and absolute sensitivity of 10^{-5}.

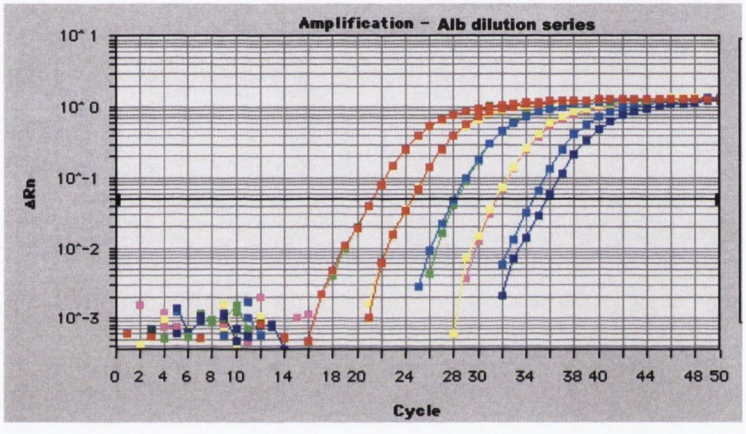
5.3.4. Reproducibility

Only reactions generating consistent and comparable results were used for RQ-PCR MRD detection.

a)



b)



c)

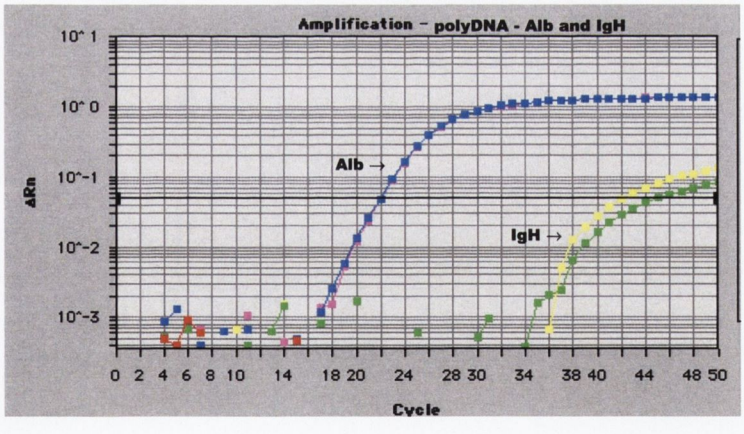


Figure 5.8. RQ-PCR of clonal IGH rearrangements (complete IGH), specificity and sensitivity test: patient's IGH dilution series with positivity detected at 10^{0} - 10^{-5} (in 10^{-5} dilution only one reaction of duplicate was positive) (panel a); patient's albumin (ALB) dilution series with positivity detected at 10^{0} - 10^{-4} (panel b); polyclonal human DNA positive for Alb and negative for IGH (IGH was only detected after the cycle 40 and therefore considered as negative) (panel c). According to the accepted criteria (Table 4.11.) the specificity range of this reaction is 10^{0} - 10^{-5}, absolute sensitivity 10^{-5}, reproducible sensitivity 10^{-4}.

5.4. RT-PCR and RQ-PCR of WT1 expression

5.4.1. Overall characteristics of RT-PCR for detection of total WT1 and its main isoforms

For RT-PCR of total *WT1*, the primers chosen for the first round involved forward (Fwd) primer WT1-A located on exon 7 and reverse (Rvs) primer WT1-B located at the end of exon 10. For the nested PCR, internal primers including Fwd primer WT1-C located on exon 7 and Rvs primer WT1-D located on exon 10 were selected (Table 5.2. and Figure 4.7.).

To identify the four main *WT1* isoforms – variants A, B, C and D, two forward and two reverse primers that can be combined into four independent sets were designed for the first round PCR, and one set of internal primers common to all *WT1* variants was designed for nested PCR. The first round PCR was performed with Fwd primer WT1-EX4/6 located on the boundary of exon 4 and 6, which allows detection of variants lacking exon 5, Fwd primer WT1-EX5 located in and detecting variants containing exon 5, Rvs primer WT1-EX9/10 spanning the boundary of exon 9 and 10 and detecting variants lacking KTS insert, and Rvs primer WT1-KTS design to bind to the KTS insert and the neighbouring sequences of exon 9 and 10, detecting variants with KTS insert. Internal primers involving Fwd primer WT1-EX6 located on exon 6 and Rvs primer WT1-EX8 on exon 8 were used for nested PCR of all variants (Table 5.2. and Figure 4.7.).

The characteristics of the optimised RT-PCR for *WT1* and its isoforms were well within the ranges required for MRD detection by BIOMED-1 protocols, giving reproducible results with high specificity and sensitivity (Figure 5.9. and 5.10.).

5.4.2. Overall characteristics of RQ-PCR for detection of total WT1 and its main isoforms

The set of primers and probe selected for RQ-PCR of total *WT1* consisted of Fwd primer WT1-Fwd located on exon 6, Rvs primer WT1-Rvs located on exon 7, and 6-FAM and TAMRA labelled probe WT1-Pr located on the exon 6 and 7 boundary. For detection of *WT1* isoforms, the following primers were employed: Fwd primer EX5-Fwd located on exon 5, Rvs primer EX5-Rvs located on exon 6 and 6-FAM and TAMRA labelled probe EX5-Pr located on the exon 5/6 boundary were used for detection of *WT1*-exon5[+], and Fwd primer KTS-Fwd on exon 9, Rvs primer KTS-Rvs spanning the end of exon 9 and the whole KTS fragment, and 6-FAM and TAMRA labelled KTS-Pr located on exon 9 were used for *WT1*-KTS[+] isoforms, respectively (Figure 4.7.).

The parameters of the RQ-PCR reactions (robustness, standard curve slope, intercept and correlation coefficient, reproducible sensitivity and specificity) fully complied with requirements for quantification of gene transcripts and MRD detection according to EAC protocols (Table 5.3.).

With WT1 plasmid calibrators the standard curves generated a slope of - 3.43 to - 3.53 and an intercept of 38.2 to 39.4 Ct. The mean Ct value obtained for 10^6 WT1 copies was 17.7 to 18.8 to ± 0.01 to 0.1. The lowest copy number used was 10^1 WT1 copies which gave positive and reproducible results in all

experiments (and all reactions performed in duplicate or triplicate) with the mean Ct 34.9 to 36.1 ± 0.6 to 0.8. With the K-562 cell line RNA dilution series, the standard curves generated a slope of - 3.34 to - 3.45 and an intercept of 21.7 to 21.9. The lowest dilution reproducibly detected was 10^{-5}.

Validation analysis indicated approximately equal efficiency of total WT1 and control gene (ABL, B2M) RQ-PCR reactions, with the absolute values of the slopes of WT1/ABL plasmid calibrator standard curve and WT1/B2M K-562 RNA dilution standard curve < 0.1 (0.055 to and 0.075 to respectively) (Figure 5.11. and 5.12.).

5.4.3. Specificity

As *WT1* expression could be detected in all haematopoietic cells and cell lines, E. coli rRNA together with no template control (sdH_2O) were used as negative controls. All RT- and RQ-PCR reactions proved to be quite specific for *WT1* detection, with neither false positive nor false negative results observed in any experiments (Figure 5.9. to 5.12.).

Table 5.2. RT-PCR for WT1 - primer sets, sizes of products and sensitivities.

RT-PCR for WT1 - primer sets, sizes of products and sensitivities		
PCR for WT1, first round and nested		
PCR reaction	Size of PCR product	Log sensitivity for K-562
A + B	483	-3
A + B → C + D	296	-5
PCR for WT1 isoforms, first round and nested		
PCR reaction	Size of PCR product	Log sensitivity for K-562
EX4/6 + EX9/10 (A)	444	-2
EX5 + EX9/10 (B)	469	-3
EX4/6 + KTS (C)	442	-2
EX5 + KTS (D)	467	-3
EX4/6 + EX9/10 → EX6 + EX8	243	-4
EX5 + EX9/10 → EX6 + EX8	243	-4
EX4/6 + KTS → EX6 + EX8	243	-4
EX5 + KTS → EX6 + EX8	243	-4

Table 5.3. Parameters of RQ-PCR for WT1.

Parameters of RQ-PCR for WT1		
RQ-PCR for total WT1		
Characteristics	Plasmid calibrators	K-562 RNA
Efficiency		
ΔRn	> 1	> 1
Standard curve slope	- 3.45	- 3.34
Standard curve intercept	39.4	21.7
Standard curve correlation coefficient	0.999	0.994
WT1/CG standard curve slope	0.055	0.075[(1)]
Sensitivity		
Reproducible sensitivity	10 molecules	- 5 log dilution
RQ-PCR for WT1/exon5[+] isoforms		
Characteristics	Plasmid calibrators	K-562 RNA
Efficiency		
ΔRn	> 1	> 1
Standard curve slope	- 3.43	- 3.44[(1)]
Standard curve intercept	38.2	21.9
Standard curve correlation coefficient	0.999	0.999
Sensitivity		
Reproducible sensitivity	10 molecules	- 5 log dilution
RQ-PCR for WT1/KTS[+] isoforms		
Characteristics	Plasmid calibrators	K-562 RNA
Efficiency		
ΔRn	> 1	> 1
Standard curve slope	- 3.53	- 3.45[(1)]
Standard curve intercept	39.0	21.7
Standard curve correlation coefficient	0.999	0.999
Sensitivity		
Reproducible sensitivity	10 molecules	- 5 log dilution
[(1)]for K-562 dilution up to - 4 log		

5.4.4. Sensitivity

Sensitivity of first round, nested PCR and RQ-PCR for *WT1* was high and comparable with that of RT-PCR and RQ-PCR for chromosomal aberrations.

Sensitivity of RT-PCR reactions was tested with 10-fold dilution series of K562 cell line RNA and positive patients' RNA. With the K562 cell line, the reproducible sensitivity reached 10^{-3} for first round and 10^{-5} for nested PCR of total *WT1*, and 10^{-2} – 10^{-3} for first round and 10^{-4} for nested PCR of *WT1* isoforms. The sensitivity for patients' samples varied according to their *WT1* expression. In those samples scored as strongly positive by first round PCR, the achieved sensitivity was 10^{-3} for the first round and 10^{-4} for the nested PCR of total *WT1* (Table 5.2., Figure 5.9. and 5.10.)

Sensitivity of RQ-PCR for *WT1* was tested with a set of plasmid calibrators, 10-fold dilution series of K562 cell line RNA and positive patients' RNA. The detected reproducible sensitivity was 10^{1} copies of plasmid calibrator and 10^{-5} dilution of K562. In patients' samples, the sensitivity again varied according to their level of *WT1* expression; in those with higher *WT1* overexpression the sensitivity reached 10^{-4} (Table 5.3., Figure 5.11. and 5.12.).

5.4.5. Reproducibility

For RT-PCR and RQ-PCR, quite consistent and comparable results were obtained between these assays in all experiments.

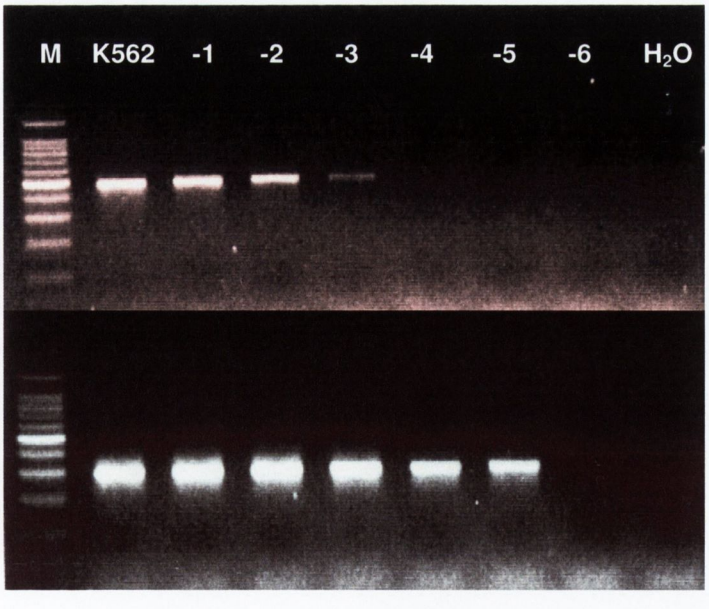


Figure 5.9. RT-PCR for total WT1, first round (upper panel) and nested (lower panel). Sensitivity test with K562 cell line dilution series (10^{-1} – 10^{-6}).

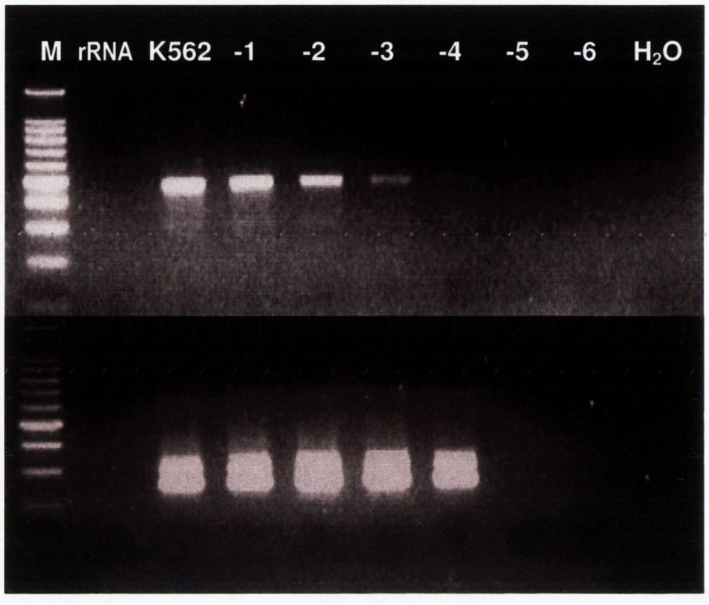


Figure 5.10. RT-PCR for WT1 isoforms, first round (upper panel) and nested (lower panel) reactions for WT1 variant D. Sensitivity test was performed with a K562 cell line dilution series (10^{-1} – 10^{-6}).

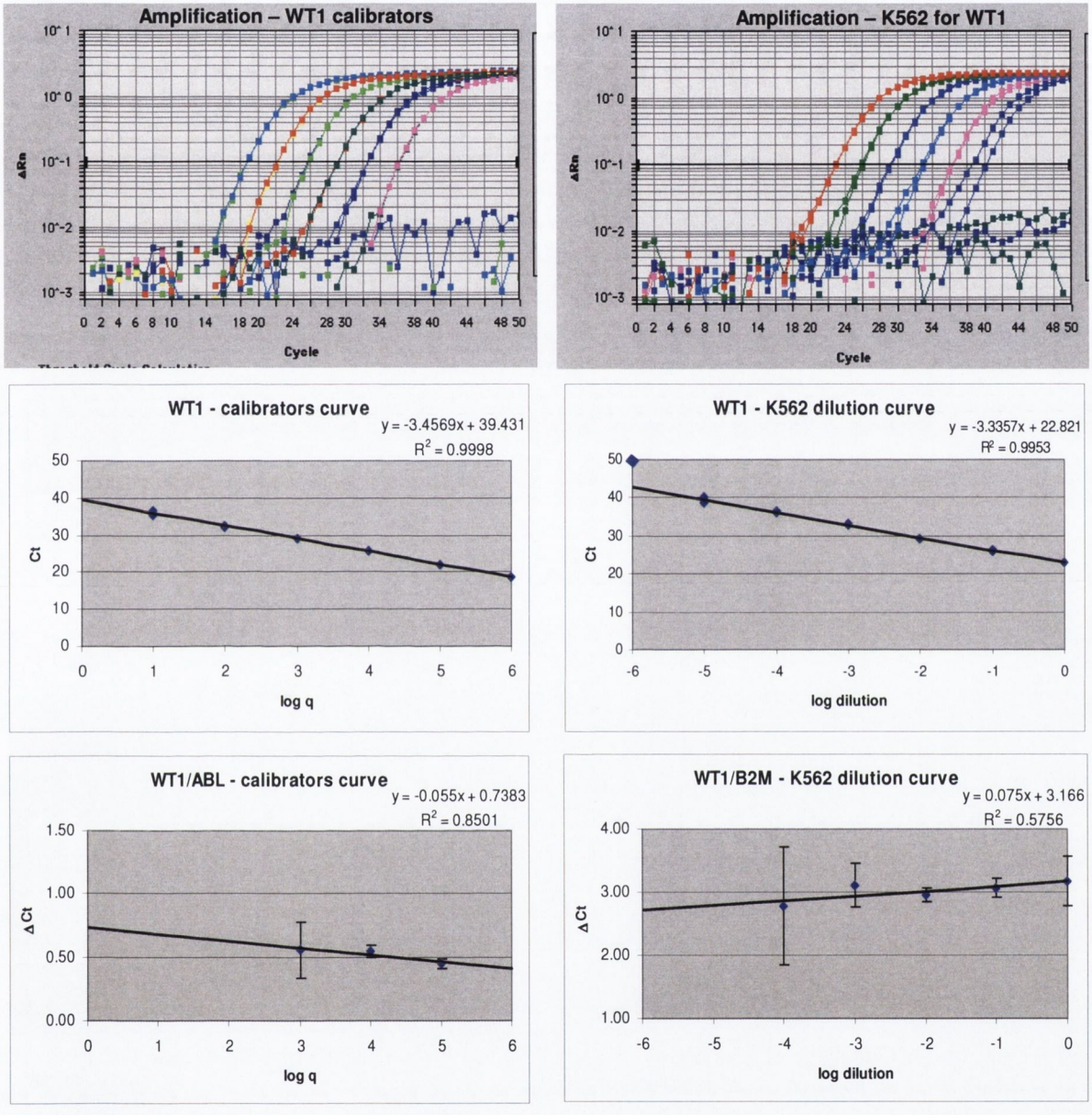


Figure 5.11. RQ-PCR for total WT1. Amplification plots (upper panels), standard curves (middle panels) and WT1/control gene validation analysis (lower panels), for plasmid calibrators (left) and K562 cell line dilution series (right).

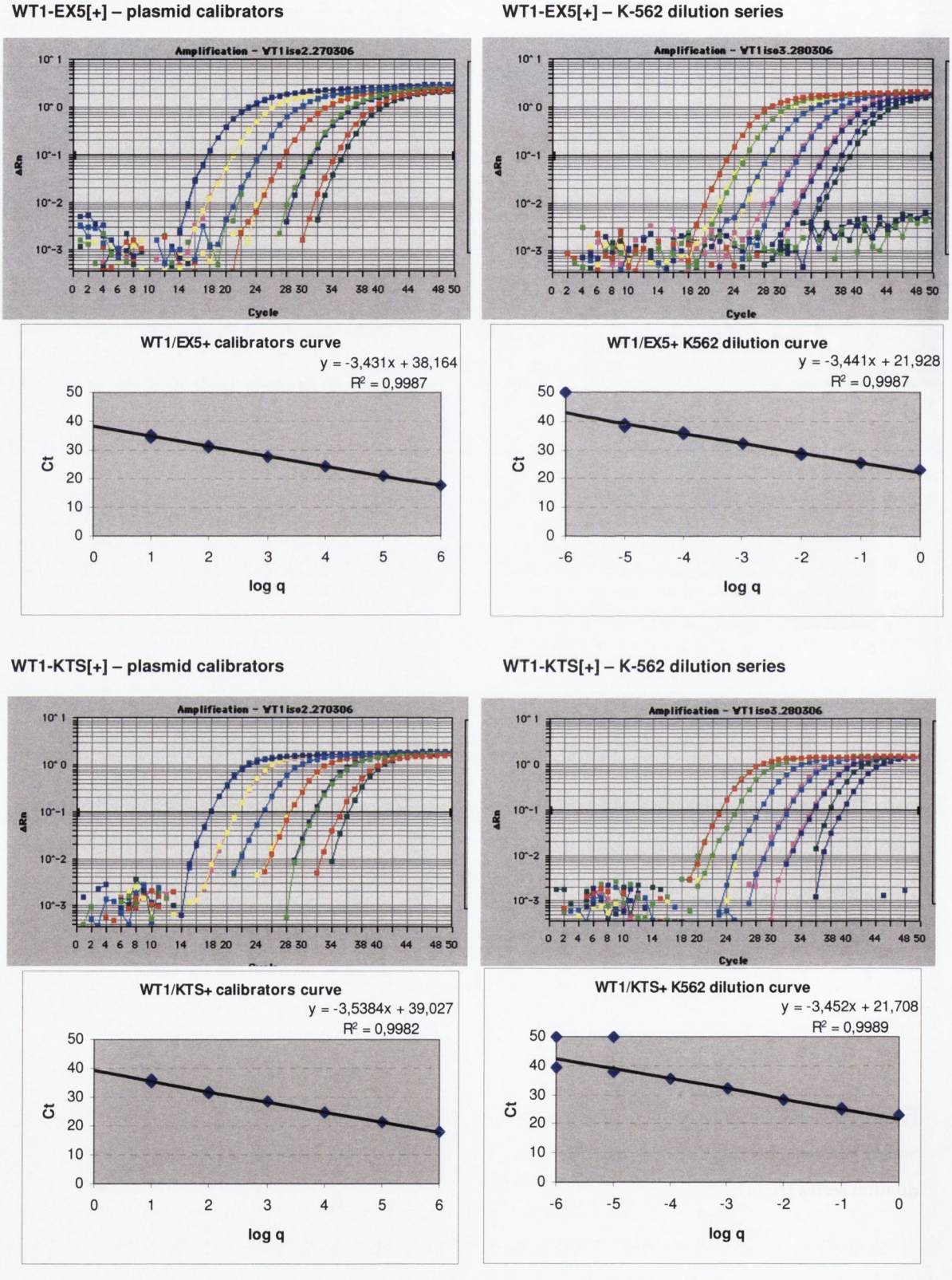


Figure 5.12. RQ-PCR for WT1 isoforms. Amplification plots and standard curves with plasmid calibrators (left) and K562 dilution series (right), for WT1-exon5[+] (upper panels) and WT1-KTS[+] (lower panels).

5.5. WT1 calibrators for real-time PCR quantification

The set of WT1 plasmid DNA calibrators prepared for absolute quantification of expression of *WT1* and its main isoforms by RQ-PCR demonstrated high performance (including purity, stability and precision of dilution) quite comparable with the other commercially purchased calibrators (Ipsogen). In repeated experiments, standard curves of WT1 plasmid calibrators showed parameters very close to those of standard curves generated with the purchased plasmid calibrators for control genes (ABL, B2M) (Table 5.3. and Figure 5.11. and 5.12.). These results suggest that these WT1 calibrators allow precise and reliable quantification of WT1 copy number by RQ-PCR and thus can be used as a standard for evaluation of *WT1* expression in clinical samples.

5.6. Detection of WT1 protein by flow cytometry and immunofluorescence microscopy

5.6.1. Flow cytometry optimisation

Results of the initial indirect intracellular staining for WT1 protein using primary anti-WT1 antibody and secondary FITC- or PE-labelled goat anti-mouse antibody confirmed that expression of WT1 protein in cells can be detected by flow cytometry (Figure 5.13.). FITC-labelled secondary antibody gave better results than PE-labelled secondary antibody and was therefore used for further experiments (Figure 5.14.). The main disadvantages of the indirect intracellular staining were increased unspecific background fluorescence and also a problematic combination with direct staining for surface markers. Staining for surface markers could be performed only after indirect staining for WT1, resulting in significantly decreased detected expression of the surface antigen due to its damage by previous fixation/permeabilisation procedures and also falsely increased detected WT1 expression due to cross-reaction of labelled secondary antibodies with mouse antibodies against surface antigens, which occurred despite extensive washing and blocking steps (Figure 5.15.). To exclude these complications and make the method more precise and faster, direct intracellular staining for WT1 was introduced.

Intracellular staining for WT1 with directly FITC-labelled anti-WT1 antibody (clone 6F-H2 DAKO) and IntraStain kit (DAKO) eliminated the technical problems mentioned above. The results and parameters of WT1 detection by this direct intracellular staining approach were quite comparable to those of established methods of FC analysis of other common intracellular antigens (Figure 5.16.). This more accurate method of FC detection of WT1 expression in haematopoietic cells revealed a new finding – repeatedly detected weak WT1 positivity in a proportion of normal peripheral blood cells that were used as a negative control because they were supposed to be negative for WT1 (Figure 5.16 and 5.17.). The partial weak WT1 positivity in peripheral blood cells was found to be only intracellular, there was no positivity when staining for surface antigens was performed with the WT1 antibody (Figure 5.16.). It was also observed in all white blood cell types (B-cells, T-cells and granulocytes), though it was less

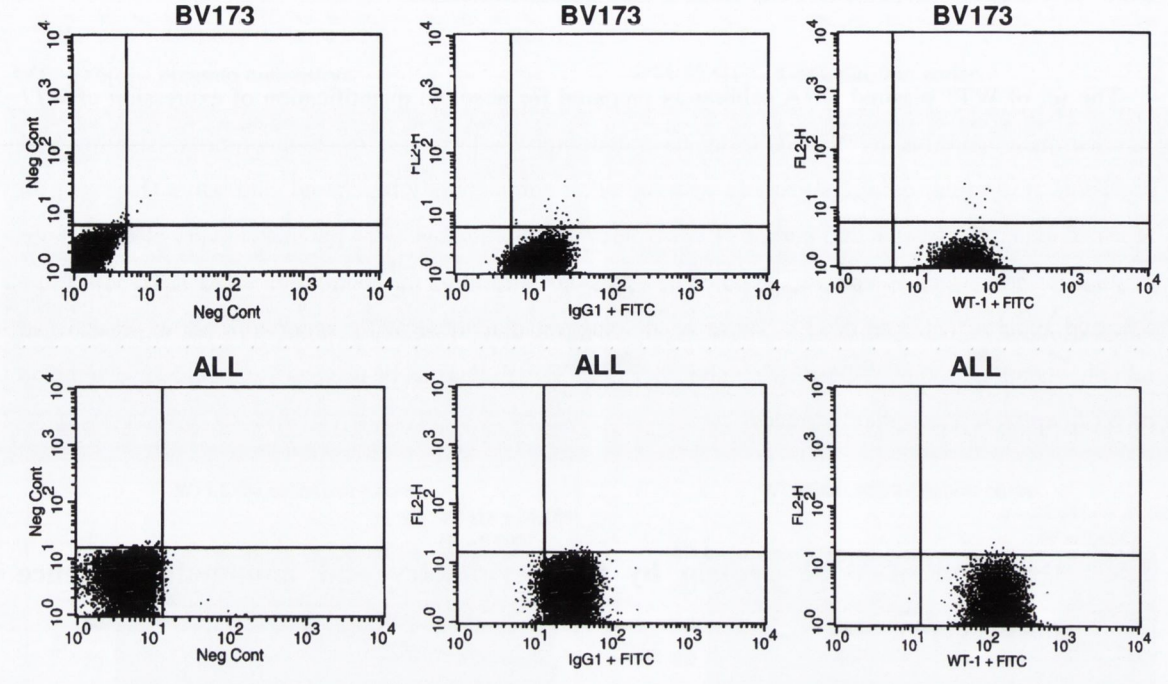


Figure 5.13. Indirect intracellular staining for WT1 using primary mouse anti-WT1 antibody (DAKO) and secondary FITC-labelled goat anti-mouse antibody (DAKO) performed on a cell line (BV173) and an ALL sample: negative control (left panels), isotype control (middle panels) and positive sample (right panels).

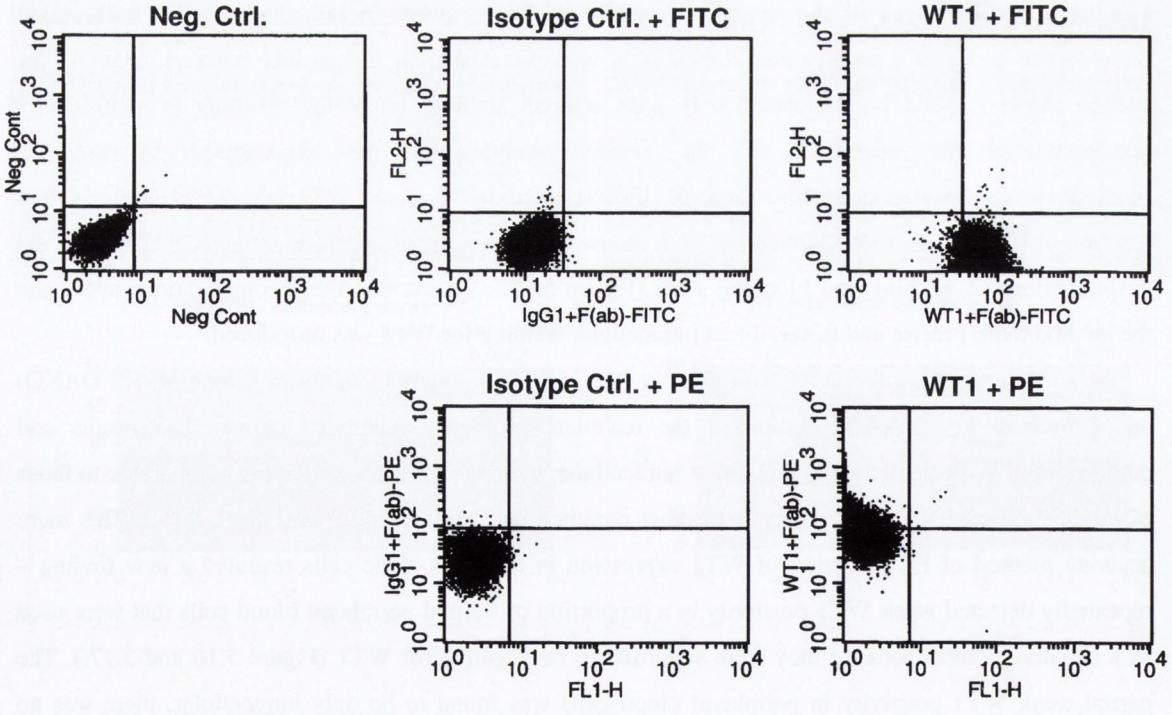


Figure 5.14. Indirect intracellular staining for WT1. Comparison of use of FITC- (upper panels) and PE-labelled (lower panels) secondary antibody (cell line MV4-11).

pronounced in B-cells and more apparent in myeloid cells (Figure 5.17.). Normal bone marrow showed weak WT1 positivity, as expected (Figure 5.16.).

A series of further optimisation experiments was then performed in order to improve the discrimination between normal cells and cells overexpressing WT1, decrease the suspicious unspecific antibody binding to WT1 negative cells and increase specific fluorescence of WT1 positive cells.

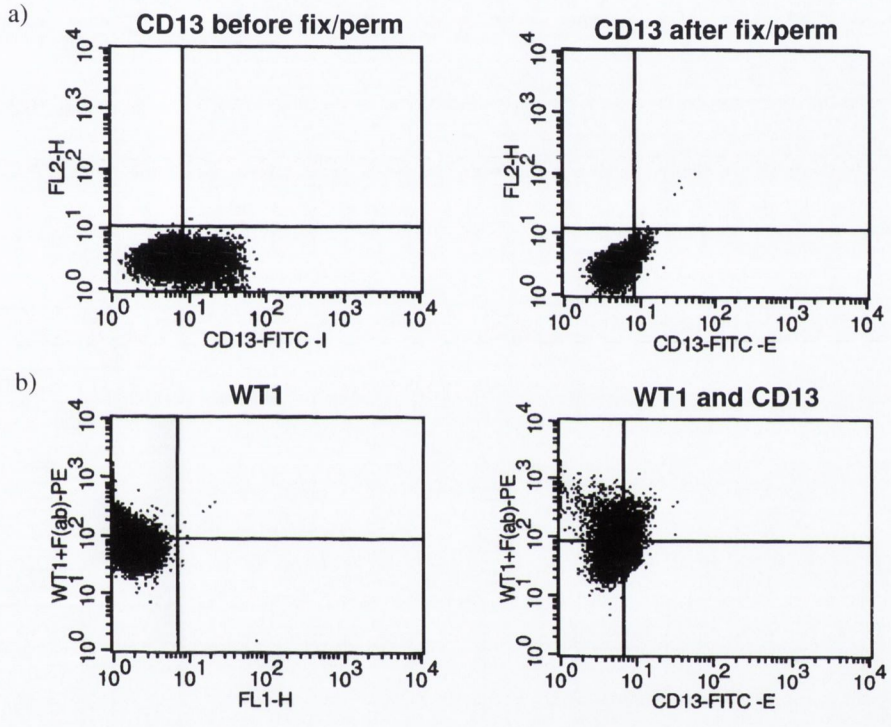


Figure 5.15. Indirect intracellular staining for WT1 followed by direct staining for a surface antigen (CD13) on a cell line (MV4-11). A surface antigen (CD13) expression before fixation and permeabilisation and its reduction after fixation and permeabilisation procedures (panel a); original WT1 expression and its false increase when staining for WT1 performed after staining for a surface antigen (CD13) – a problem caused by cross-reaction of secondary anti-mouse antibody (panel b).

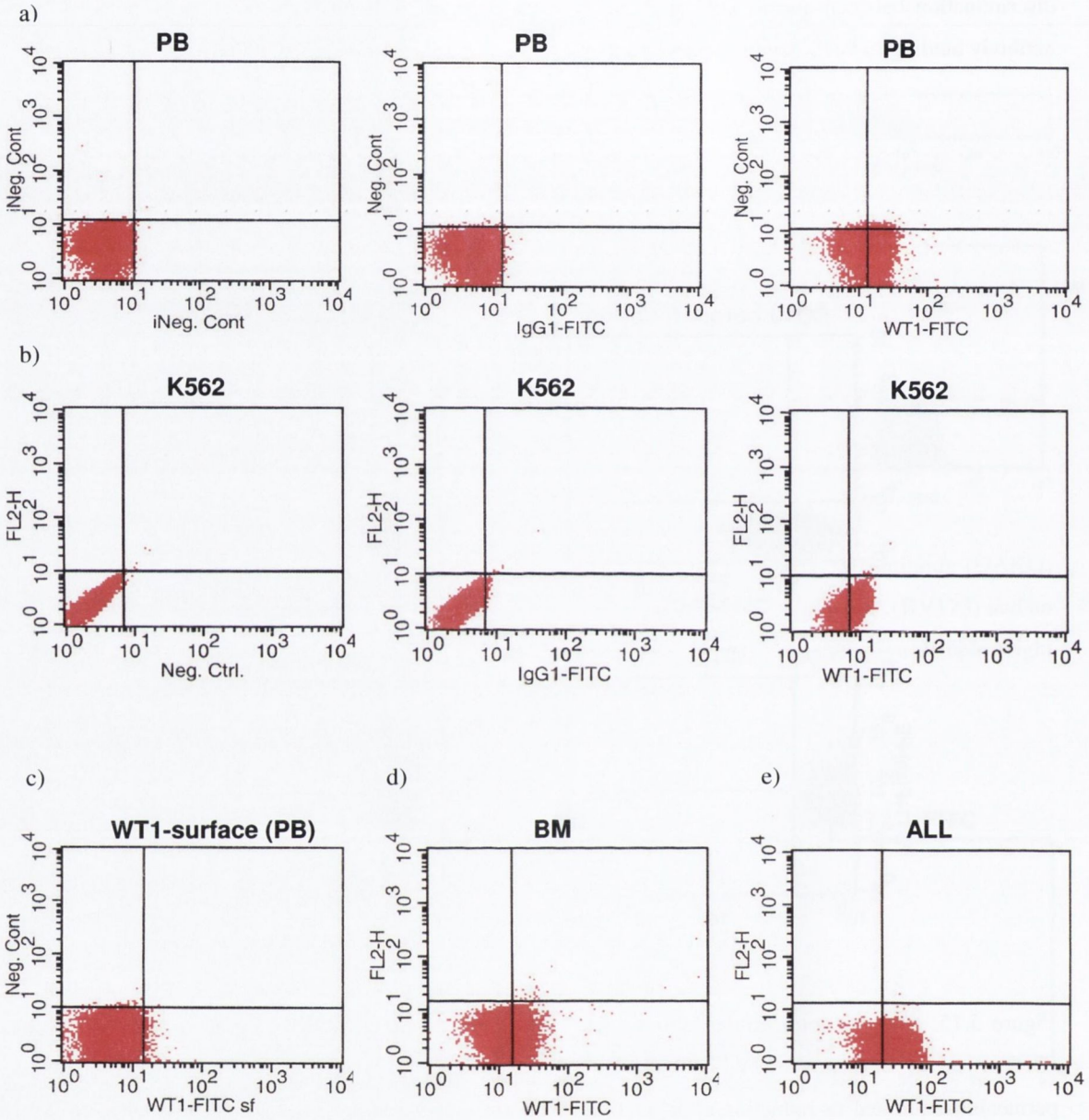


Figure 5.16. Direct intracellular staining for WT1. Normal peripheral blood (panels a), K562 cell line (panels b), normal bone marrow (panel d) and ALL bone marrow (panel e); surface staining for WT1 on normal peripheral blood cells (panel c).

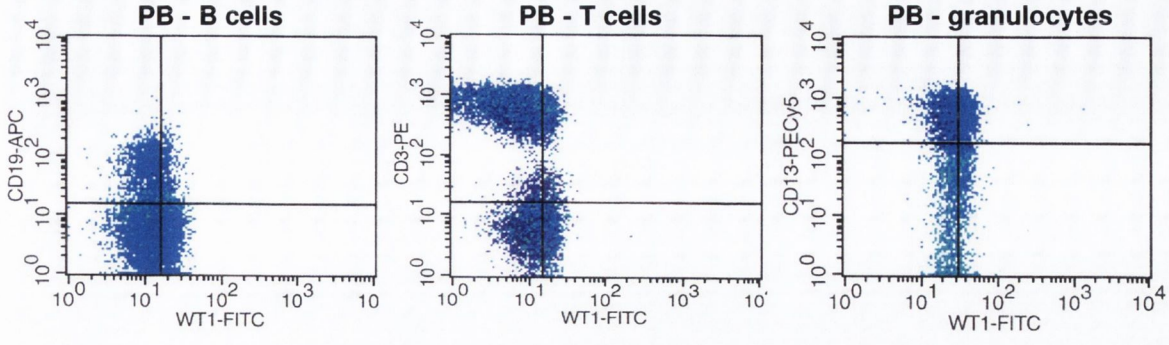


Figure 5.17. FC detected WT1 protein expression in subsets of normal peripheral white blood cells (B- and T-lymphocytes and granulocytes).

5.6.2. Characteristics of the optimised flow cytometry method

After extensive testing of a number of different methods of cells fixation and permeabilisation for intracellular staining, various washing and blocking steps, use of different clones of anti-WT1 antibody and different labelling fluorochrome, the method giving the most accurate results was finally determined. This involved anti-WT1 mouse monoclonal antibody clone WLM04 (NeoMarkers) labelled with AlexaFluor488 (Molecular Probes) in amount optimised by titration (5 µl per 10^6 cells) using direct intracellular staining with Fix and Perm kit (Caltag) (Figure 5.18.). In comparison with the original direct intracellular staining method, this optimised method showed slightly reduced WT1 positivity in normal peripheral blood cells and slightly increased fluorescence intensity of positive controls (K562 cell line or acute leukaemia samples), offering therefore improved discrimination between normal and WT1 overexpressing cells. The improvement appeared to be more related to the optimised staining technique than to the clone and fluorochrome labelling of the anti-WT1 antibody.

In this optimised set up, flow cytometry enabled measurement of levels of WT1 protein expression in haematopoietic cells (Figure 5.18.). WT1 expression may be quantified by percentage of positive cells and their mean fluorescence intensity. However, as the detected differences in WT1 expression in normal peripheral blood, normal bone marrow, acute leukaemia bone marrow and cell lines were only quantitative, within relatively limited ranges of values, and with no clear separation of positive (WT1 overexpressing) and negative (normal) cell population, flow cytometric detection of WT1 expression was not further employed for routine detection of MRD in clinical samples.

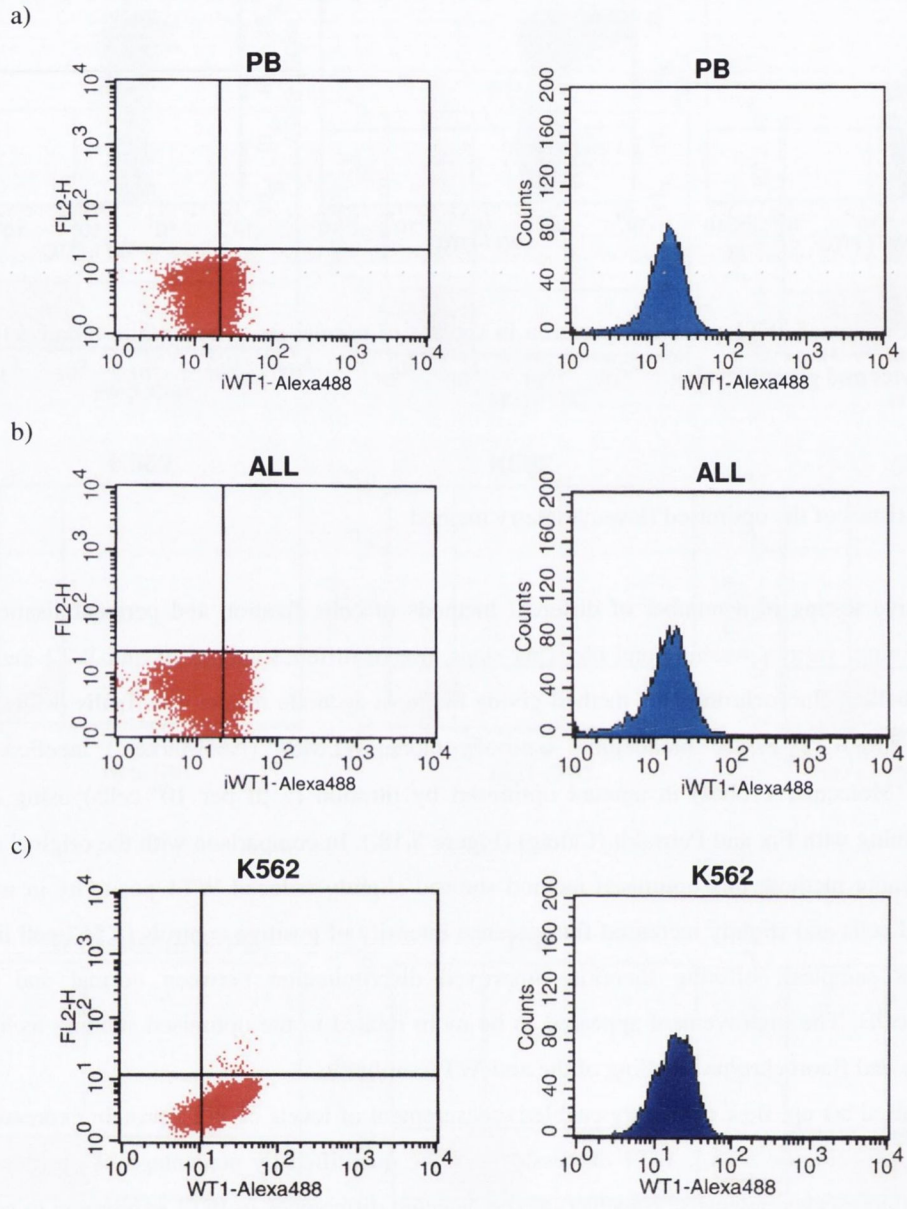


Figure 5.18. Detection of WT1 protein expression levels in haematopoietic cells by flow cytometry. Normal peripheral blood (panel a), ALL bone marrow (panel b) and K562 cell line (panel c).

5.6.3. Detection of WT1 by immunofluorescence microscopy

Detection of WT1 protein by immunofluorescence microscopy was performed at several stages of flow cytometric experiments on WT1 as a source of reference, to confirm or clarify certain results of FC.

Indirect staining with primary anti-WT1 antibody (clone 6F-H2) and secondary FITC-labelled goat anti-mouse antibody (both DAKO) was able to detect WT1 protein expression in the nuclei of positive cells (K562), but there was a considerable unspecific background fluorescence detected in the cytoplasm of all stained cells, including isotype controls, despite all washing and blocking techniques applied (Figure 5.19.).

Optimised direct staining for WT1 protein with AlexaFluor488 labelled anti-WT1 antibody (clone WLM04, NeoMarkers) gave significantly improved results. Nuclei of positive cells (K562) showed strong specific fluorescence, the degree of unspecific background of negative cells was very low. This staining also revealed granular and diffuse perinuclear and cytoplasmic positivity of a proportion of normal bone marrow cells and, in smaller extent, normal peripheral blood cells and K562 cells. This positivity seemed to be specific and more but not exclusively confined to cells of myeloid lineage (Figure 5.19.).

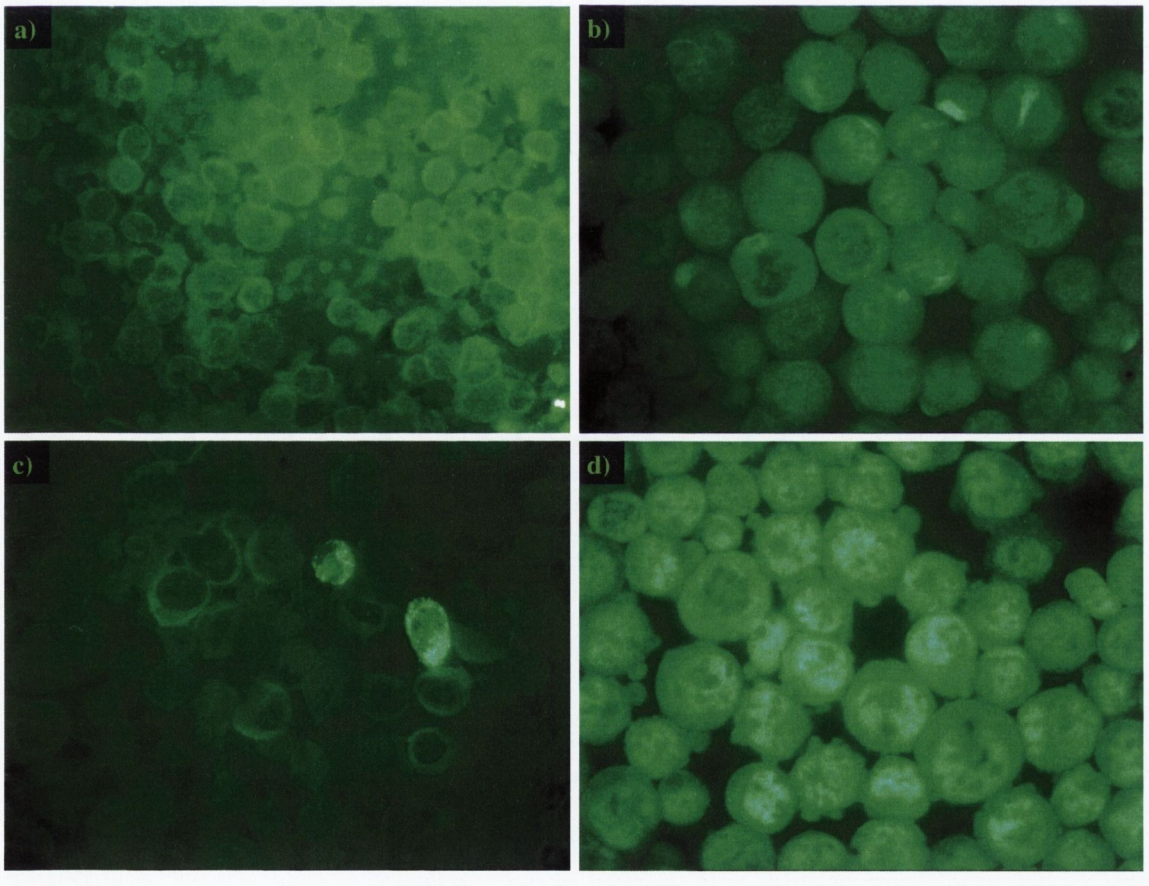


Figure 5.19. Detection of WT1 protein in haematopoietic cells by immunofluorescence microscopy. Indirect staining with primary anti-WT1 antibody and secondary FITC-labelled goat anti-mouse antibody of normal peripheral blood (panel a) and K562 cell line (panel b). Direct staining with AlexaFluor488 labelled anti-WT1 antibody of normal bone marrow (panel c) and K562 cell line (panel d).

5.7. Detection and monitoring of MRD in childhood acute leukaemia by flow cytometry immunophenotyping

5.7.1. Detection of LAPs suitable for flow cytometry MRD monitoring in diagnostic samples of childhood acute leukaemia patients

Flow cytometry immunophenotyping for MRD detection was performed in 18 out of 25 patients with childhood acute leukaemia (14 B-ALL, 2 T-ALL, 2 AML). In the remaining 7 patients, their diagnostic sample was either not obtained at all or there were insufficient cells for flow cytometry analysis.

In all patients who had FC performed, one or more LAPs suitable for MRD detection were identified (Table 5.4.).

The number of different LAPs detected in a patient's diagnostic sample ranged from 1 to 4 (mean 2). Only 1 LAP suitable for MRD monitoring was found in both T-ALL patients. However, the LAP TdT/c3/7/3, present in one of them, is known to be a stable and reliable MRD marker and thus could be itself sufficient for MRD monitoring in T-ALL. In all B-ALL and AML patients, 2 or more different LAPs were identified and used as independent MRD markers (Table 5.5.).

Of the large panel of antibody combinations that were used to screen diagnostic samples for expression of different LAPs, a relatively limited group of LAPs types could be found in all the diagnostic samples tested (Table 5.5. and Figure 5.20.). In B-ALL patients, LAP CD58/10/34/19 was present in all except one patient samples, two other frequent LAPs included CD38/34/45/19 and CD66/10/34/19, each present in over one third of the samples. Further LAPs detected in B-ALL were CD13/10/34/19, CD21/10/34/19, CD34/33/45/19, CD65/10/34/19 and m7.1/CD19. In T-ALL patients, LAPs TdT/cCD3/7/3 and CD8–/4–/7/3 were found. AML patients expressed LAPs HLA-DR/CD33/45/117, CD34/11b/45/117, CD13/33/45/117, CD7/13/45/117 and CD41/33/45/34.

The number and types of LAPs identified in a diagnostic sample of every patient were recorded. All follow-up samples of this patient were then analysed for presence of these LAPs as MRD markers.

5.7.2. Detection of MRD by flow cytometry in follow-up samples of patients with childhood acute leukaemia

The presence and level of MRD in patients' follow-up samples was detected by flow cytometry analysis of the samples for the presence and percentage of cells expressing the patient's characteristic LAPs. The MRD was assessed by FC in 16 patients (in the remaining 2 patients no follow-up samples were obtained).

Table 5.4. LAPs identified in diagnostic samples of patients with childhood acute leukaemia.

FC detection of LAPs for MRD monitoring						
UPN	Dg.	No. of LAPs	LAP1	LAP2	LAP3	LAP4
1	B-ALL	2	58/10/34/19	13/10/34/19		
2	B-ALL	3	58/10/34/19	13/10/34/19	65/10/34/19	
3	B-ALL	3	58/10/34/19	66/10/34/19	38/34/45/19	
4	B-ALL	2	38/34/45/19	58/10/34/19		
5	B-ALL	2	58/10/34/19	34/33/45/19		
7	B-ALL	3	38/34/45/19	66/10/34/19	58/10/34/19	
9	B-ALL	2	38/34/45/19	m7.1/19		
10	B-ALL	2	58/10/34/19	38/34/45/19		
12	B-ALL	2	58/10/34/19	66/10/34/19		
13	B-ALL	2	58/10/34/19	66/10/34/19		
14	B-ALL	2	58/10/34/19	38/34/45/19		
16	B-ALL	3	58/10/34/19	66/10/34/19	21/10/34/19	
18	B-ALL	2	58/10/34/19	21/10/34/19		
20	B-ALL	2	58/10/34/19	13/10/34/19		
22	T-ALL	1	TdT/c3/7/3			
23	T-ALL	1	8–/4–/7/3			
24	AML	4	DR/33/45/117	34/11b/45/117	13/33/45/117	41/33/45/34
25	AML	4	DR/33/45/117	34/11b/45/117	13/33/45/117	7/13/45/117

(UPN – unique patient's number, Dg – diagnosis)

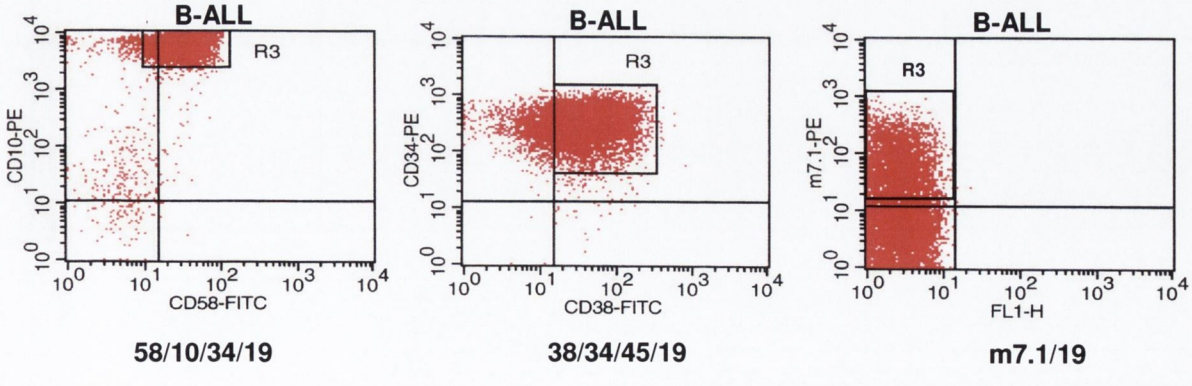


Figure 5.20. Example of LAPs detected in B-ALL diagnostic samples: CD58/10/34/19 (UPN10; left panel), CD38/34/45/19 (UPN9; middle panel) and m7.1/19 (UPN9; right panel).

Table 5.5. Numbers and types of LAPs detected in diagnostic samples and used for FC MRD monitoring.

Number and types of LAPs detected in diagnostic samples		
Number of LAPs		
B-ALL (n = 14)	2 LAPs: 10 pts (71 %)	
	3 LAPs: 4 pts (29 %)	
T-ALL (n = 2)	1 LAP: 2 pts	
AML (n = 2)	4 LAPs: 2 pts	
Type of LAP	No. of patients	% of patients
B-ALL (n = 14)		
CD58/10/34/19 (quantitative)	13	93
CD38/34/45/19 (quantitative)	6	43
CD66/10/34/19 (cross-lineage myeloid)	5	36
CD13/10/34/19 (cross-lineage myeloid)	3	21
CD21/10/34/19 (maturation	2	14
asynchronous)	1	7
CD34/33/45/19 (cross-lineage myeloid)	1	7
CD65/10/34/19 (cross-lineage myeloid)	1	7
m7.1/CD19 (ectopic)		
T-ALL (n = 2)		
TdT/cCD3/7/3 (ectopic)	1	(50)
CD8–/4–/7/3 (maturation	1	(50)
asynchronous)		
AML (n = 2)		
HLA-DR/CD33/45/117 (maturation	2	(100)
asynchronous)	2	(100)
CD34/11b/45/117 (maturation	2	(100)
asynchronous)	1	(50)
CD13/33/45/117 (maturation	1	(50)
asynchronous)		
CD7/13/45/117 (cross-lineage T-cell)		
CD41/33/45/34 (maturation		
asynchronous)		

MRD presence and level at different time-points

Of 14 ALL patients analysed, 8 (57 %) had undetectable MRD in day 29 samples and remained MRD negative during the whole follow-up (UPN1, 3, 4, 10, 13, 14, 18, 22). MRD was detected in day 29

samples of 4 patients (29 %; UPN2, 5, 7, 9). Two of them showed low MRD levels (0.01 – 0.099 %); one of them achieved MRD negativity in the next follow-up samples from week 7 – 12 (UPN2), the other died before next sample was collected (UPN9). Two patients had significant MRD levels (0.1 – 0.99 %) on day 29. Of these, one achieved MRD negativity (UPN5) and one had still detectable low MRD level in the following samples from week 7 – 12 (UPN7). In 2 (14 %) patients, there were no available samples from day 29 and week 7 – 12 (UPN12, 16). All later samples of ALL patients were MRD negative, no MRD was detected in samples after week 12 (Table 5.6. and Figure 5.21.).

Of the 2 AML patients, only one follow-up sample from each of them was received. MRD was detectable in both of these samples from week 7 - 12; one patient (UPN24) had medium MRD level (0.1 – 0.99 %), the other (UPN25) had very high levels exceeding the amount detected at diagnosis (Table 5.6.).

Table 5.6. Results of MRD monitoring by flow cytometry in a patient cohort.

Detection of MRD by flow cytometry			
Time point	Day 29	Week 7 - 12	Month 6 - 12
ALL: *(n = 14)*	negative: 8 pts	negative: 7 pts no sample available: 1 pt	negative: 12 pts no sample available: 1 pt
	positive: 4 pts MRD levels: 0.01 – 0.099 %: 2 pts 0.1 – 0.99 %: 2 pts	negative: 1 pt died: 1 pt negative: 1 pt positive: 1 pt MRD level: 0.01 – 0.099 %	
	no sample available: 2 pts	no sample available: 2 pts	
AML: *(n = 2)*		positive: 2 pts MRD levels: 0.1 – 0.99 %: 1 pt > 100 %: 1 pt	no samples available: 2 pts

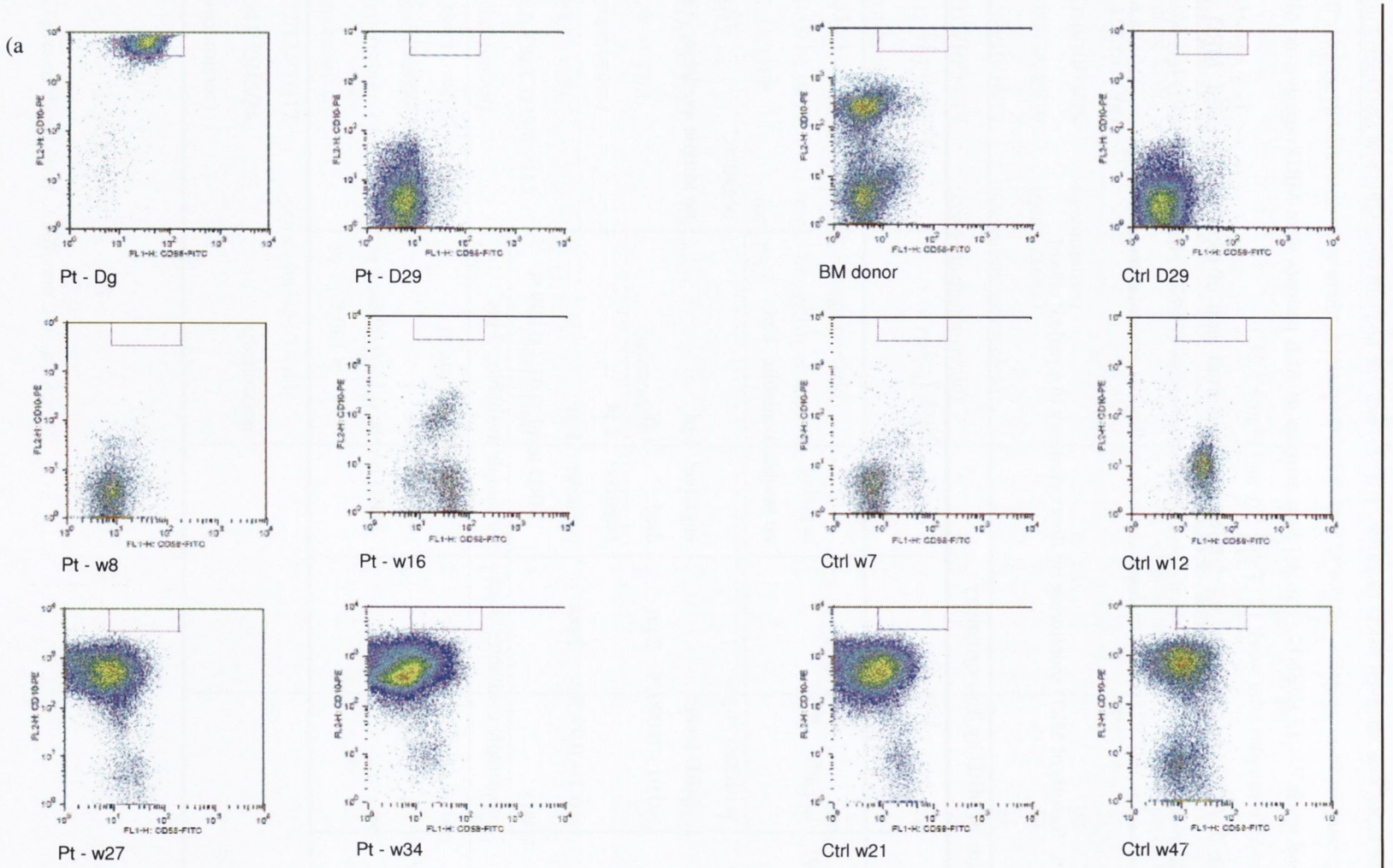


Figure 5.21. Example of MRD monitoring by FC in B-ALL patients: a patient with the LAP CD58/10/34/19 MRD negative from D29 (UPN10; panels a), and a patient with the LAP CD38/34/45/19 MRD positive on D29 and w12, further negative (UPN7; panels b). Patients' samples (the left panels) are compared with controls – normal bone marrow and ALL patients MRD negative by PCR (the right panels). (w7 – w47 - time points: week 7 – week 47) – cont.

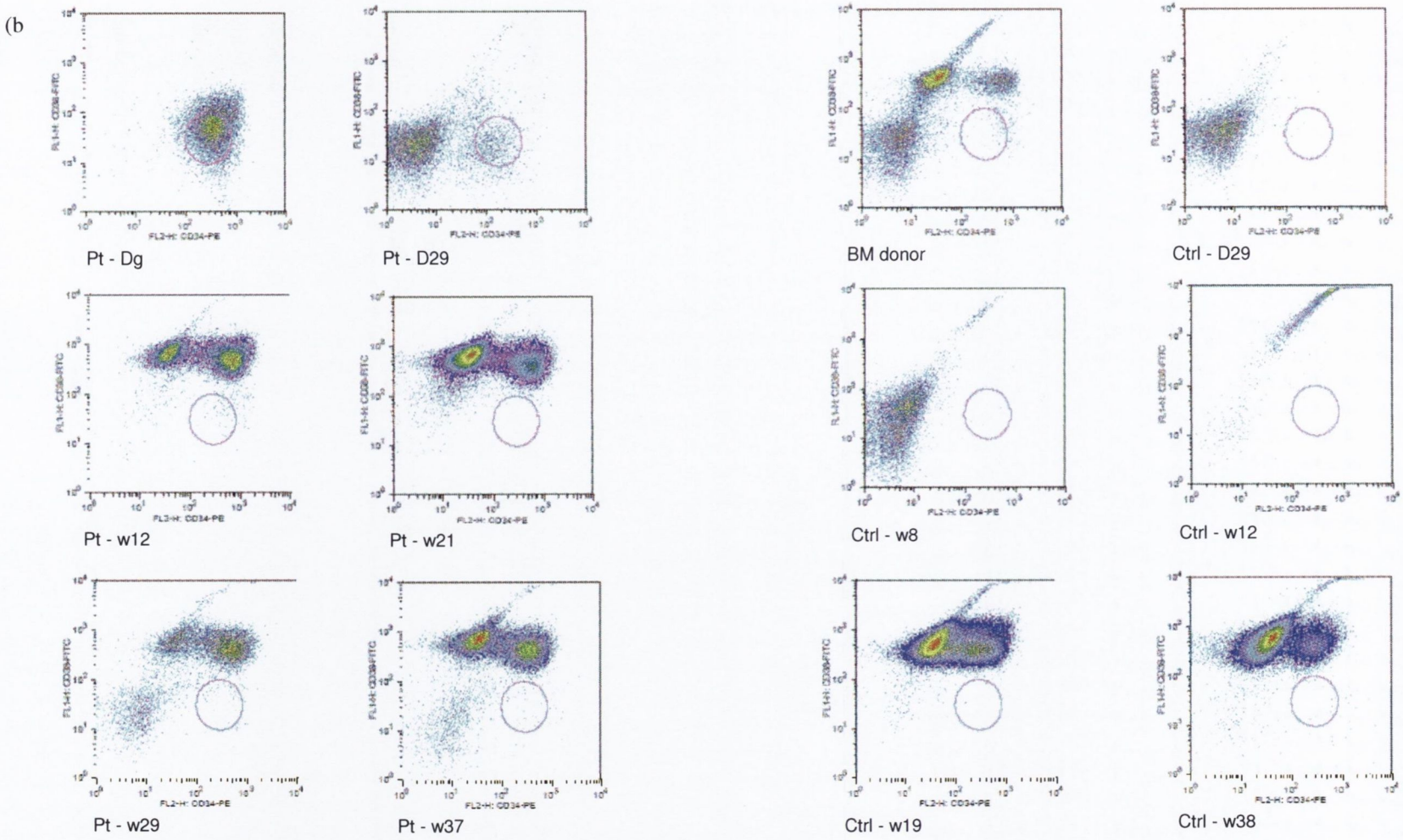


Figure 5.21. Example of MRD monitoring by FC in B-ALL patients: a patient with the LAP CD58/10/34/19 MRD negative from D29 (UPN10; panels a), and a patient with the LAP CD38/34/45/19 MRD positive on D29 and w12, further negative (UPN7; panels b). Patients' samples (the left panels) are compared with controls – normal bone marrow and ALL patients MRD negative by PCR (the right panels). (w7 – w47 - time points: week 7 – week 47).

Correlation of MRD detected in the same patients by different LAPs

In the patients where 2 or more LAPs were identified and used for MRD detection, the results obtained from analysis of the 2 most reliable LAPs were correlated in between (69 pairs of LAPs from 16 patients and at various time-points). A good overall correlation was achieved with $p < 0.0001$ (Spearman rank test), and bias 5.5 (Bland-Altman comparison) (Figure 5.22.).

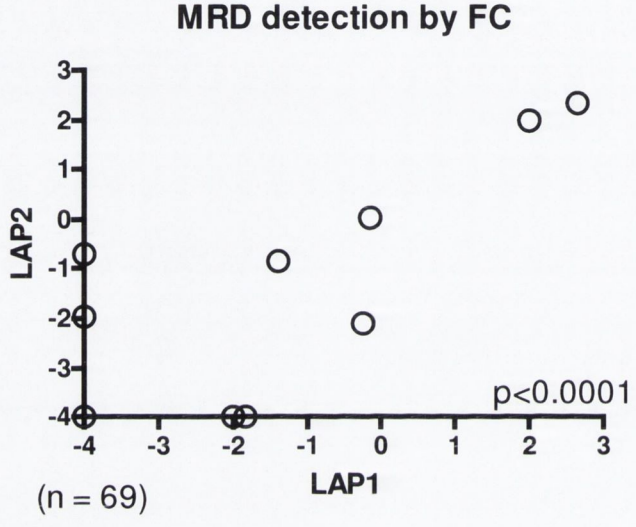


Figure 5.22. Correlation of results of FC MRD detection using 2 LAPs as independent targets.

5.8. Detection and monitoring of MRD in childhood acute leukaemia by RT-PCR and RQ-PCR of fusion genes transcripts

5.8.1. PCR detection of chromosomal aberrations in diagnostic samples of childhood acute leukaemia patients

RT-PCR detection of chromosomal aberrations was performed in all 25 patients with childhood acute leukaemia (20 B-ALL, 3 T-ALL, 2 AML). With the screening PCR for relevant aberrations, 8 clearly positive results and one weakly positive finding were detected in B-ALL patients. No chromosomal aberrations were found in patients with T-ALL and AML (Table 5.7.).

All the 8 patients (UPN1, 2, 4, 5, 10, 13, 16, 20) with clear positivity of first round PCR expressed the translocation t(12;21) *TEL-AML1*. The results were verified by confirmatory PCR (Figure 5.23.).

In 1 patient with B-ALL (UPN9), a weak positivity was found in first round PCR for t(4;11) *MLL-AF4*, with a band of a bigger size (between 700 – 800 bp) than would be expected of normal products of this reaction. The result of confirmatory PCR for t(4;11) *MLL-AF4* was negative for this patient. This

patient was therefore considered to be negative for t(4;11) *MLL-AF4* translocation; however, there could potentially be another *MLL* gene rearrangement present in this patient (Figure 5.24.).

The diagnostic and all follow-up samples of patients with confirmed chromosomal aberrations were then analysed by RQ-PCR to quantify the level of the fusion gene expression and determine the MRD status.

Table 5.7. Chromosomal aberrations detected in diagnostic samples of patients with childhood acute leukaemia.

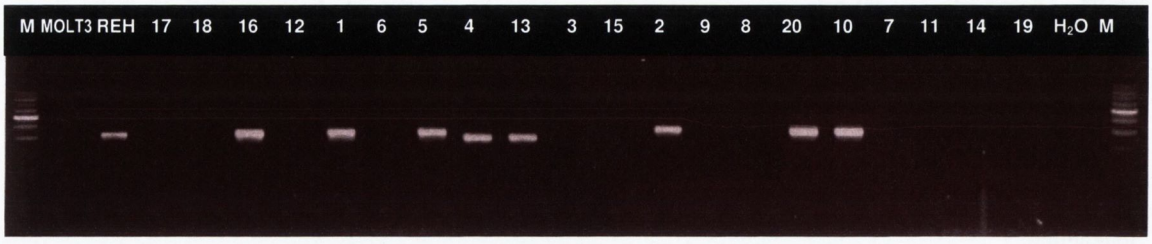
Chromosomal aberrations in diagnostic samples of patients with childhood acute leukaemia		
Chromosomal aberration	No. of patients	% of patients
B-ALL (n = 20)		
t(9;22) BCR-ABL p190	0	0
t(9;22) BCR-ABL p210	0	0
t(1;19) E2A-PBX1	0	0
t(4;11) MLL-AF4	0	0
t(12;21) TEL-AML1	8	40
standard transcript	4	20
variant transcript (- 39 bp)	2	10
both transcripts	2	10
T-ALL (n = 3)		
microdel(1p) SIL-TAL1	0	0
AML (n = 2)		
t(8;21) AML1-ETO	0	0
t(15;17) PML-RARA	0	0
inv(16) CBFB-MYH11	0	0

5.8.2. Detection of MRD by RQ-PCR of fusion gene transcripts from chromosomal aberrations in follow-up samples of patients with childhood acute leukaemia

The presence and level of MRD in follow-up samples of 7 patients (in 1 patient there were no follow-up samples available) was detected by RQ-PCR of the fusion gene transcripts.

Only 2 (29 %) patients had undetectable MRD on day 29 and in all next follow-up samples (UPN1 and 4). Four patients (57 %) were MRD positive on day 29, 3 of them (UPN2, 5, 10) with very low MRD levels (< 0.01 %), one (UPN13) with a low MRD level (0.01 – 0.099 %). One of the patients (UPN2) with very low MRD positivity on day 29 remained very weakly positive (< 0.01 %) in week 7 - 12 sample. All further follow-up samples were MRD negative. In the one patient (UPN16) with no day 29 sample available, MRD negativity was confirmed since week 7 - 12 (Table 5.8., Figure 5.25. and 5.26.).

a)



b)

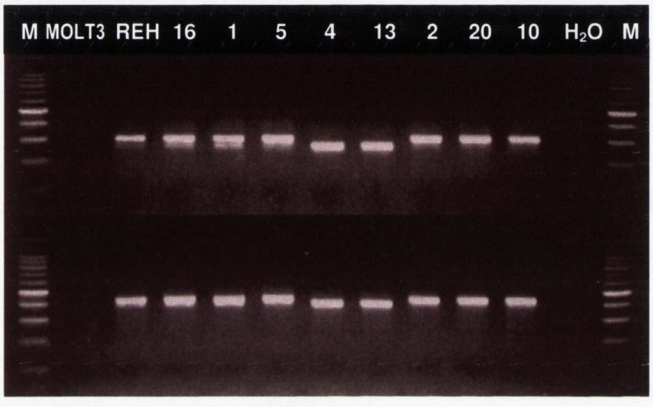


Figure 5.23. Screening RT-PCR for t(12;21) TEL-AML1 (panel a); confirmatory RT-PCR for t(12;21) TEL-AML1 for the positively screened patients (panel b, lower) together with their results from the previous screening reaction (panel b, upper) (M – size marker, MOLT3 – cell line, negative control, REH – cell line, positive control, numbers – patients' UPNs).

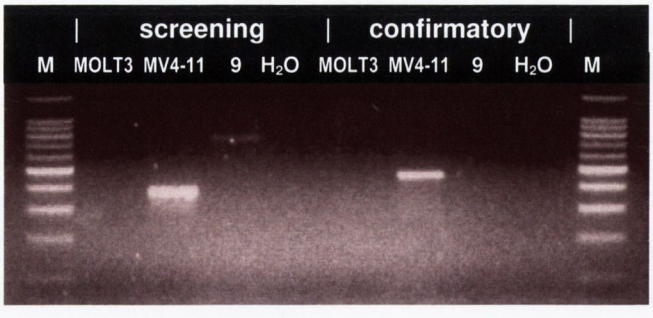


Figure 5.24. Screening and confirmatory RT-PCR for t(4;11) MLL-AF4 in a patient with a weakly positive band of abnormal size in the screening PCR reaction and negative confirmatory PCR (M – size marker, MOLT3 – cell line, negative control, MV4-11 – cell line, positive control, 9 – patient UPN9).

Table 5.8. MRD monitoring by RQ-PCR of chromosomal aberrations.

Detection of MRD by TEL-AML1 translocation in B-ALL			
Time point	Day 29	Week 7 - 12	Month 6 - 12
ALL: *(n = 7)*	negative: 2 pts	negative: 2 pts	negative: all 7 pts
	positive: 4 pts MRD levels: 0.01 – 0.099 %: 1 pt < 0.01 %: 3 pts	negative: 1 pt	
		negative: 2 pts positive: 1 pt MRD level: < 0.01 %	
	no sample available: 1 pt	negative: 1 pt	

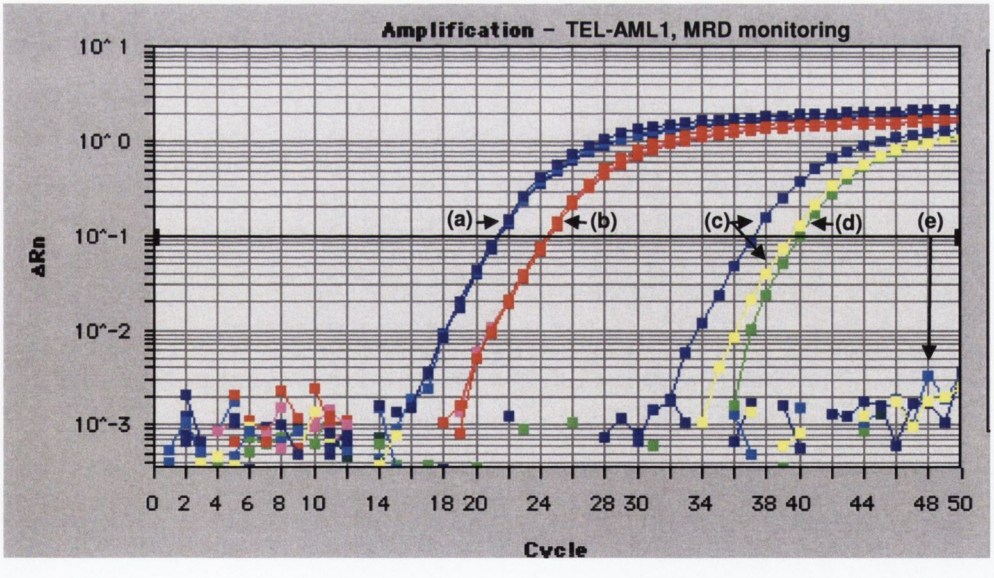


Figure 5.25. Example of MRD monitoring by RQ-PCR of chromosomal aberrations in a B-ALL patient expressing t(12;21) TEL-AML1 translocation (UPN2). The diagnostic sample (a), day 8 sample with high levels of MRD (b), day 29 and week 7 samples with very low levels of MRD (c) and (d), and week 19 sample, MRD negative (e).

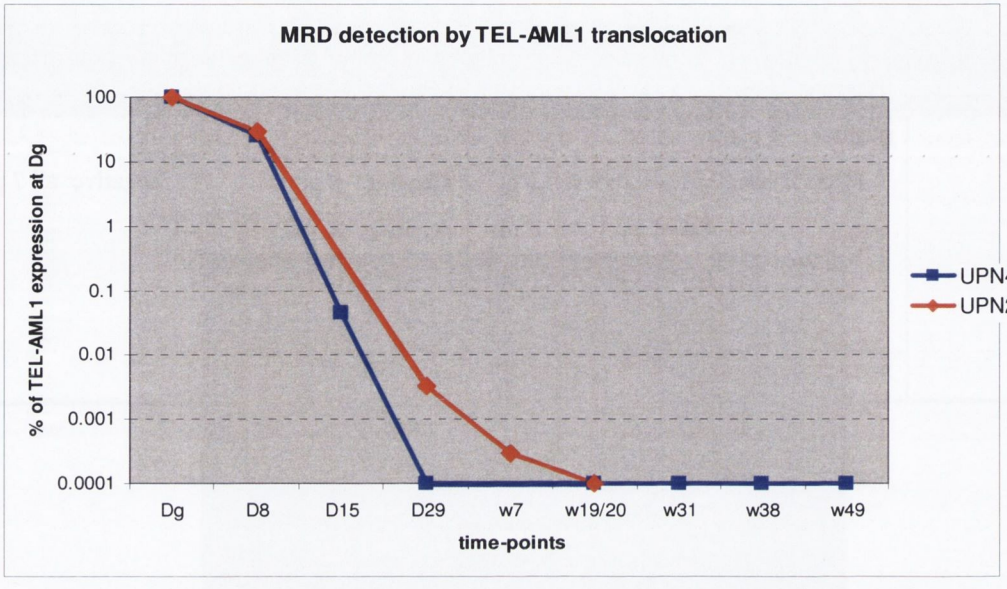


Figure 5.26. Example of MRD monitoring by RQ-PCR of chromosomal translocation TEL-AML1. A patient (UPN4; blue) with a fast response to the chemotherapy – MRD negativity achieved from day 29; a patient (UPN2; red) with slower response to the therapy – MRD negativity achieved only in later sample from week 19.

5.9. Detection and monitoring of MRD in childhood acute leukaemia by PCR and RQ-PCR of clonal IG/TCR rearrangements

5.9.1. Identification of clonal IG/TCR targets in diagnostic samples of childhood acute lymphoblastic leukaemia patients and design of patient-specific RQ-PCR systems for detection of clonal IG/TCR rearrangements

PCR screening for clonal *IG/TCR* rearrangements was performed in 14 out of 23 patients with ALL (in the remaining 9 patients, no diagnostic and/or follow-up DNA was available). Their initial screening was carried out with multiplex reactions for IGH-FR1-3, IGH-DH incomplete rearrangements and IGK-Kde (B-ALL), TCRD and TCRG rearrangements (T-ALL). Among the 13 B-ALL patients, 12 patients showed mono- or biclonal positivity in IGH (11 complete, 1 incomplete rearrangements) and 9 patients in IGK-Kde. The only one T-ALL patient showed monoclonal positivity in TCRD (Figure 5.27., Table 5.9. and 5.10).

All the patients were sequenced (in case of the presence of biclonal bands each band was cut out of the gel and sequenced separately). In one patient (UPN10) that appeared to have a monoclonal IGH complete rearrangement, no accurate sequence was obtained from repeated sequencing of products of IGH-FR1, 2 and 3. This patient was negative for IGK-Kde rearrangement and had no more DNA available for further screenings. As we were not able to identify any clonal rearrangement in this patient, MRD monitoring by PCR of *IG/TCR* clonal rearrangements was not performed in this case.

In all the other patients, good quality sequences were obtained, junctional regions and N-nucleotides identified and ASO primers designed and tested. Optimal ASO primers suitable for MRD detection were found for all IGH and TCRD clonal targets but only for 5 out of 9 IGK rearrangements. The remaining 4 patients with IGK rearrangements had either long CG sequences in their junctional regions or very short junctional regions with only one or two N-nucleotides and here we did not manage to design an ASO primer that would give acceptable results.

Finally, 13 of the 14 patients had *IG/TCR* clonal rearrangements identified and specific RQ-PCR for their detection designed. Of the 12 B-ALL patients, 2 independent clonal targets were found in 6 of them (46 %), and 1 clonal target in the other 6 patients. In all these patients with only 1 identified *IG/TCR* target, other methods of MRD detection were applicable and used (eg. FC, PCR of chromosomal aberrations). According to the type of the *IG/TCR* clonal targets, the most common were IGH complete rearrangements (11; 61 %), followed by IGK-Kde (5; 28 %) and IGH incomplete rearrangements (2; 11 %). In the T-ALL patient, TCRD rearrangement as the only one target was detected and used (Table 5.9. and 5.10.).

The diagnostic and all follow-up samples of these patients were then analysed by RQ-PCR to quantify the level of expression of the *IG/TCR* clonal target and determine the MRD status.

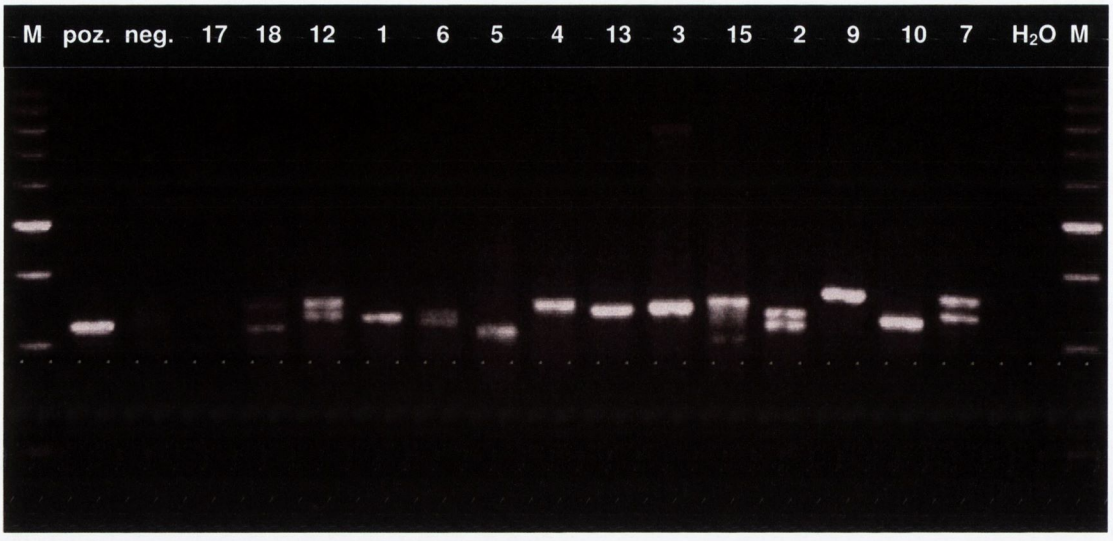


Figure 5.27. Clonality screening with IGH-FR1 multiplex PCR reactions (InVivoScribe) and visualisation on MetaPhor agarose gel: M - size marker, poz. - pozitive control (cell line), neg. - negative control (polyclonal human DNA), numbers - screened patients' UPNs: patients UPN1, 13, 3, 9 and 10 show clear monoclonal bands and were sequenced directly from this PCR; patients UPN18, 12, 6, 5, 4, 2 and 7 were re-screened with FR2 reaction and/or in case of the presence of two clear bands these were cut out of the gel and the extracted PCR product was used for sequencing; patients UPN17 and 15 were considered negative; patient UPN17 was screened for other Ig/TCR rearrangements, patient UPN15 in whom no diagnostic sample was available was screened from a pre-diagnostic sample with low amount of blasts but excluded from further Ig/TCR analysis.

Table 5.9. IG/TCR clonal rearrangements detected in diagnostic samples and used for MRD monitoring.

IG/TCR clonal rearrangements detected in diagnostic samples and used for MRD monitoring		
Number of IG/TCR clonal targets		
B-ALL (n = 13)	2: 6 pts (46 %) 1: 6 pts (46 %) 0: 1 pt (8 %)	
T-ALL (n = 1)	1: 1 pt	
Type of IG/TCR clonal rearrangements	No. of targets	% of targets
B-ALL		
IGH complete	11	61
IGH incomplete	2	11
IGK-Kde	5	28
T-ALL		
TCRD	1	

Table 5.10. Patients's specific IG/TCR clonal rearrangements – junctional regions and the used ASO primers.

Patients' specific junctional regions and designed ASO primers			
UPN	IG/TCR rearrangement	Junctional region	Junctional region sequences and designed ASO primers *)
1	IGH complete IGK-Kde	VH3-D6 VK-Kde	GCTGTGTATTACTGTGCG*GTTCTAGAGCTCTAGGGC*AGC TATTACTGTCTACAAGATTACAATTACCCT*GGGGG*GTGG
2	IGH complete	VH1-D2	GAGATCTGAGGACA*CGGATTCCT*AGGATATTGTAGTGGT
3	IGH complete	D3-JH5	GAG*ATCACCAC*GTATTACTATGGTTCGGGGA*CAATT*AAC
4	IGH complete	VH3-D2	CGAAAGA*TGGGGGTATTAGGGAT*TTGTAGTAGTACCAGC
5	IGK-Kde	VK-Kde	AGCCTGAAGATTTTGCAGTGTATTACTGTC*CCA*GGAGC
6	IGH complete IGK-Kde	VH6-D4-JH4 VK-Kde	ACGGCTGTGT*CTTTCGTCGTTT*TGGTAAC*CTGGGAT*CTT CAAATTATTACTGTCTGCAGAGTAAGAATT*CTTC*GGAGC
7	IGH complete	D3-JH6	CTGTG*GG*TACGATATTTTGACTGGTTAT*CCCACCCC*TTA
9	IGH complete	D2-JH6	TTTGTACTAATGGTGTATGC*CCTTCCGACACTCCCG*ATT
12	IGH complete IGK-Kde	VH1-D3-JH3 VK-Kde	AGA*AAC*GTATTACTATGATAGTAGTGG*CGACTGATG*GCT ATTACTGTCTGCAGAGTAAGAATTTTCCTC*AAG*GCCCT
13	IGH complete IGK-Kde	VH3-D5-JH6 VK-Kde	TGTGTATTACTGTGCGAGA*TC*ATAGTGGCTACGA*AA*TAC GGGGTTTATTACTGCATGCAAGGTACACAC*CTA*CCTAG
17	IGH incomplete IGH incomplete	D4-JH6 D5-JH6	CTCCTGCTGTGTGACTACGGTGGTAAC*CCCACTGGG*TAC GTCAGACTGTGGTGGATACAGCTATGGTTA*AGGG*TACTA
18	IGH complete IGH complete	VH4-D6-JH5 VH3-D2	CCGTGTATTACTGTGCGAG*TTGGACAT*GCAGCTCG*G*TGG GTGTATTACTGTGCGA*AAAGGCTTCGG*GGATATTGTACT
23	TCRD	DV2-DD3	GTGA*ACCGAGGACAAAGGCTGTTA*ACTGGGGG*CTGT*ACA

*) N-nucleotides of the junctional regions in red and italic, sequences of ASO primers underlined

5.9.2. Detection of MRD by RQ-PCR of clonal IG/TCR rearrangements in follow-up samples of patients with childhood acute lymphoblastic leukaemia

MRD was monitored by RQ-PCR of clonal *IG/TCR* rearrangements in follow-up samples of 12 out of 13 patients in whom the specific individual PCR reactions were established. One patient died before day 29 (UPN23) but in his samples available from day 8 and day 15 the levels of MRD were very high.

MRD presence and level at different time-points

In 7 (58 %) patients (UPN1, 3, 4, 5, 6, 9, 18), MRD was undetectable from day 29; these patients remained MRD negative in all later samples. Five (42 %) patients showed MRD positivity on day 29, 2 (UPN7 and 12) in a medium level (0.1 – 0.99 %), 2 (UPN2, 13) in a low level (0.01 - 0.099 %) and 1 (UPN17) in a very low level (< 0.01 %). The two patients with the medium MRD level remained MRD positive till week 21 and 26, respectively, and only after this time point achieved MRD negativity. Of the two patients with the low MRD level, 1 (UPN2) was further positive on week 7 - 12, all other samples of these two patients were negative. The patient with the very low MRD level was negative on week 7 - 12 and further follow up samples with one exception - a very weak positivity on week 34 (Table. 5.11. and Figure 5.28.).

Table 5.11. MRD monitoring by RQ-PCR of IG/TCR clonal rearrangements.

Detection of MRD by IG/TCR clonal rearrangements in ALL			
Time point	Day 29	Week 7 - 12	Month 6 - 12
ALL: *(n = 13)*	negative: 7 pts	negative: 6 pts died: 1 pt	negative: 6 pts
	positive: 5 pts MRD levels: 0.1 – 0.99 %: 2 pts	positive: 1 pt MRD level: 0.01 - 0.099 % no sample available: 1pt	positive (w21 resp. w26, MRD level: < 0.01 %), further negative: 2 pts
	0.01 - 0.099 %: 2 pts	negative: 1 pt positive: 1 pt MRD level: < 0.01 %	negative: 2 pts
	< 0.01 %: 1 pt	negative: 1 pt	positive (on w34, MRD level: < 0.01 %), other time-points negative: 1 pt

Correlation of MRD detected in the same patients by two independent clonal IG/TCR targets

In the patients where 2 independent *IG/TCR* clonal rearrangements were identified and used for MRD monitoring, their results were correlated in between (41 pairs from 6 patients at various time-points). An excellent correlation was achieved with $p < 0.0001$ (Spearman rank test), and bias 1.1 (Bland-Altman comparison) (Figure 5.29.).

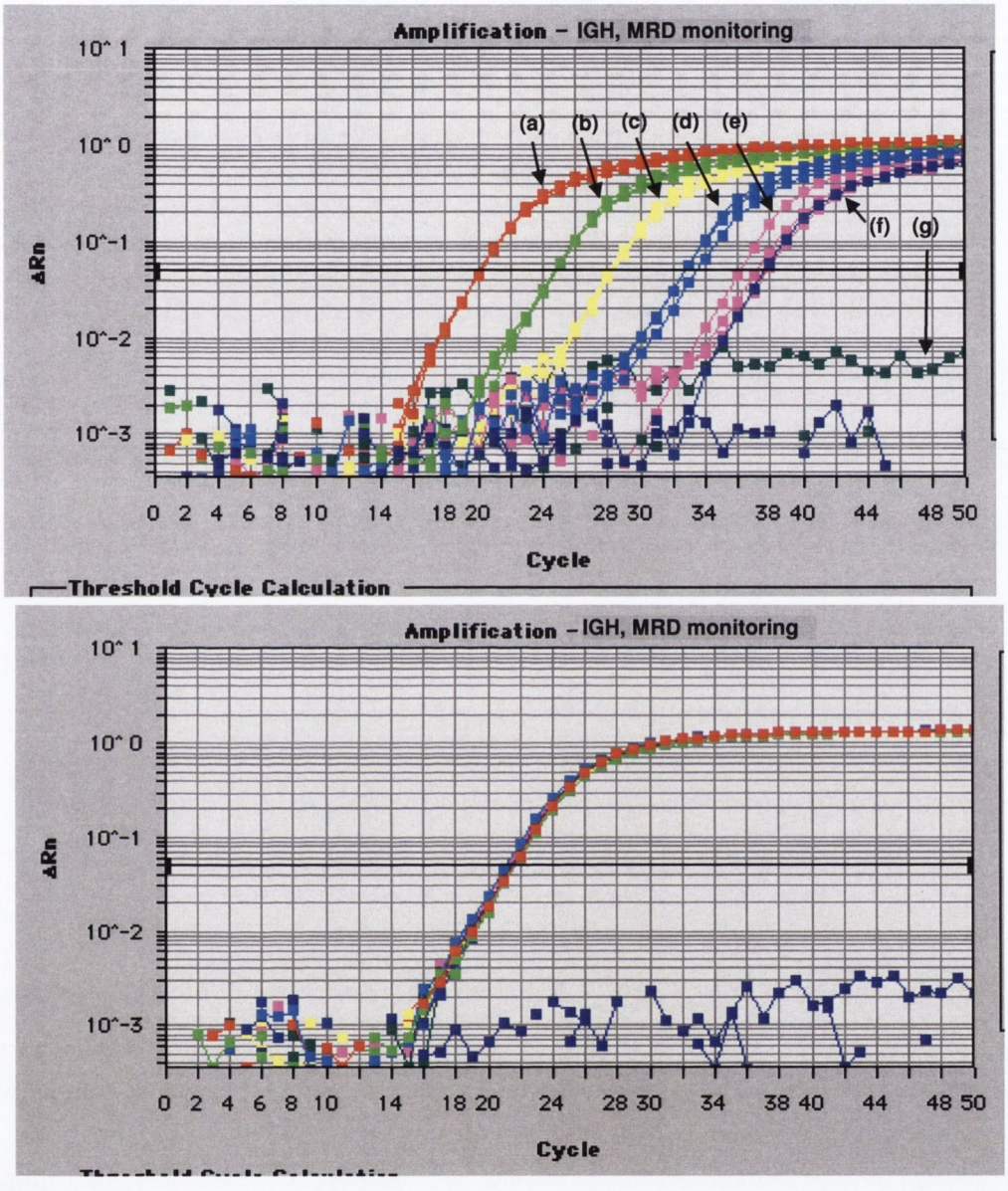


Figure 5.28. Example of MRD monitoring by RQ-PCR of IGH clonal rearrangement in a B-ALL patient (UPN7). Upper panel: the diagnostic sample (a), follow-up samples with decreasing levels of MRD - day 15 sample (b), day 29 (c), week 13 (d) and week 21 samples (e), week 29 sample with a single very low positivity of a triplicate reaction below the defined threshold of sensitivity, therefore considered as negative (f), and week 37 sample, MRD negative (g). Lower panel: detection of the control gene (albumin) in these samples.

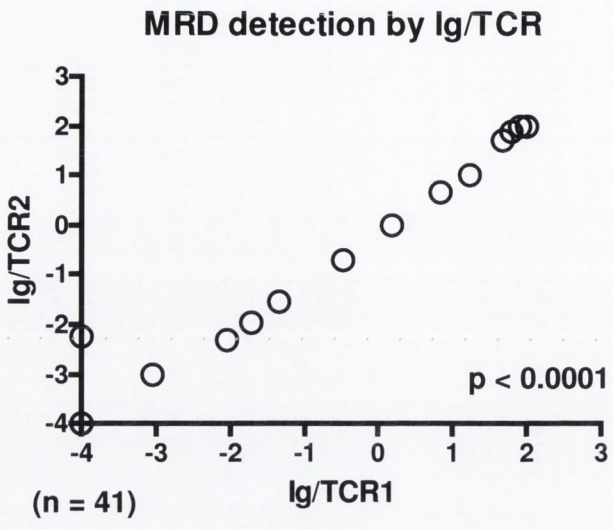


Figure 5.29. Correlation of results of RQ-PCR MRD detection using 2 independent IG/TCR targets.

5.10. Detection and monitoring of MRD in childhood acute leukaemia by different methods, their correlation in between and with clinical data

5.10.1. Results and correlation of MRD detection by flow cytometry and PCR methods

Results of MRD detection by all methods

MRD was monitored in 18 of the 25 patients enrolled in the study. The remaining 7 patients either did not have a diagnostic sample sent to our lab at all or in a very small, insufficient amount or did not have any follow-up samples available. In all patients with a sufficient amount of diagnostic sample, MRD could be detected by at least one method and 2 independent targets.

Of the 16 ALL patients, MRD could be detected with only one method in 4 of them (25 %), using FC in 2 patients (UPN14, 22) and PCR of *IG/TCR* rearrangements in the other 2 patients (UPN6, 17), each representing 13 %. Two different methods, FC and PCR, were applicable in 7 patients in total (44 %), FC and PCR of *IG/TCR* rearrangements in 5 patients (31 %; UPN3, 7, 9, 12, 18), FC and PCR of chromosomal aberrations in 2 patients (13 %; UPN10, 16). Detection of MRD by all three different methods, FC + PCR of *IG/TCR* rearrangements + PCR of chromosomal aberrations could be performed in 5 patients (31 %; UPN1, 2, 4, 5, 13).

In both AML patients (UPN24, 25), MRD could be monitored by FC only (Table 5.12.).

Table 5.12. Methods of MRD detection applicable in childhood acute leukaemia.

Methods of MRD detection in childhood acute leukaemia		
Type of leukaemia and methods	No of pts	% of pts
ALL (n = 16)		
FC only	2	13
PCR-IG/TCR only	2	13
FC + PCR-IG/TCR	5	31
FC + PCR-translocations	2	13
FC + PCR-IG/TCR + PCR-translocations	5	31
AML (n = 2)		
FC only	2	(100)

Among ALL patients, 7 (44 %) were MRD negative from day 29 in all further samples (UPN1, 3, 4, 6, 14, 18, 22). Eight (50 %) patients were MRD positive on day 29, 2 (UPN7, 12) in a medium level (0.1 – 0.99 %), 3 (UPN2, 9, 13) in a low level (0.01 - 0.099 %) and 3 (UPN5, 10, 17) in a very low level (< 0.01 %). The two patients with the medium MRD level achieved MRD negativity only after week 21 and 26, respectively. One of the 3 patients with the low MRD level died before next follow-up (UPN9), one (UPN2) was MRD positive also on week 7 – 12 and then negative, the last one (UPN13) was negative beginning week 7 – 12. All 3 patients with the very low MRD level were negative on week 7 - 12 and in further follow up samples with one exception - a very weak positivity on week 34 in one of them (UPN17). One patient (UPN16) had no day 29 sample available (6 %) and all his later samples were MRD negative. (Table. 5.13. and Figure 5.30.)

In 11 patients with ALL, early samples from day 15 were available and analysed. MRD was present in a very low level (< 0.01 %) in 2 of them (18 %) and all were negative on day 29 (UPN1, 18). Five patients (45 %) showed a low MRD level (0.01 – 0.099 %) in these samples, and 3 of them were MRD negative (UPN3, 4, 6) while 2 positive on day 29 (UPN10, 17). One patient (9 %; UPN7) had MRD of a high level (1 – 9.9 %) and 3 (27 %; UPN12, 13, 23) of very high levels (>10 %). Three of them remained MRD positive in significant levels in their day 29 samples, one patient died before day 29 (UPN23).

The 2 AML patients were both MRD positive in samples from week 7 - 12; one patient (UPN24) with a medium MRD level (0.1 – 0.99 %), the other (UPN25) with a very high level exceeding the amount detected at diagnosis (Table 5.13.).

Table 5.13. MRD detection - summarised results from FC and PCR methods.

Detection of MRD by FC and PCR			
Time point	Day 29	Week 7 - 12	Month 6 - 12
ALL: *(n = 16)*	negative: 7 pts	negative: 6 pts no sample available: 1 pt	negative: 6 pts no sample available: 1 pt
	positive: 7 pts MRD levels: 0.1 – 0.99 %: 2 pts	positive: 1 pt MRD level: 0.01 - 0.099 % no sample available: 1 pt	positive (w21 resp. w26, MRD level: < 0.01 %), further negative: 2 pts
	0.01- 0.099 %: 3 pts	negative: 1 pt positive: 1 pt MRD level: < 0.01 % died: 1 pt	negative: 2 pts
	< 0.01 %: 3 pts	negative: 3 pts	negative: 2 pts positive (on w34, MRD level: < 0.01 %), other time-points negative: 1 pt
	no sample available: 1 pts	negative: 1 pt	negative: 1 pt
AML: *(n = 2)*		positive: 2 pts MRD levels: 0.1 – 0.99 %: 1 pt > 100 %: 1 pt	no samples available: 2 pts

Correlation of MRD results detected by different methods

There was an overall very good correlation between the results of MRD detection by different methods, in terms of evaluating MRD positivity/negativity as well as estimating MRD levels. The agreement was excellent between the two PCR methods, PCR of chromosomal aberrations and of clonal *IG/TCR* rearrangements (5 patients, 34 pairs of values), with $p < 0.0001$ (Spearman rank test) and bias 1.6 (Bland-Altman comparison) (Figure 5.31.); and very good between FC and PCR methods (13 patients, 92 pairs of values) - $p < 0.0001$ (Spearman rank test) and bias 3.1 (Bland-Altman comparison) (Figure 5.32.). However, the PCR methods show slightly higher sensitivity for detection of very low MRD levels (below 0.1 %).

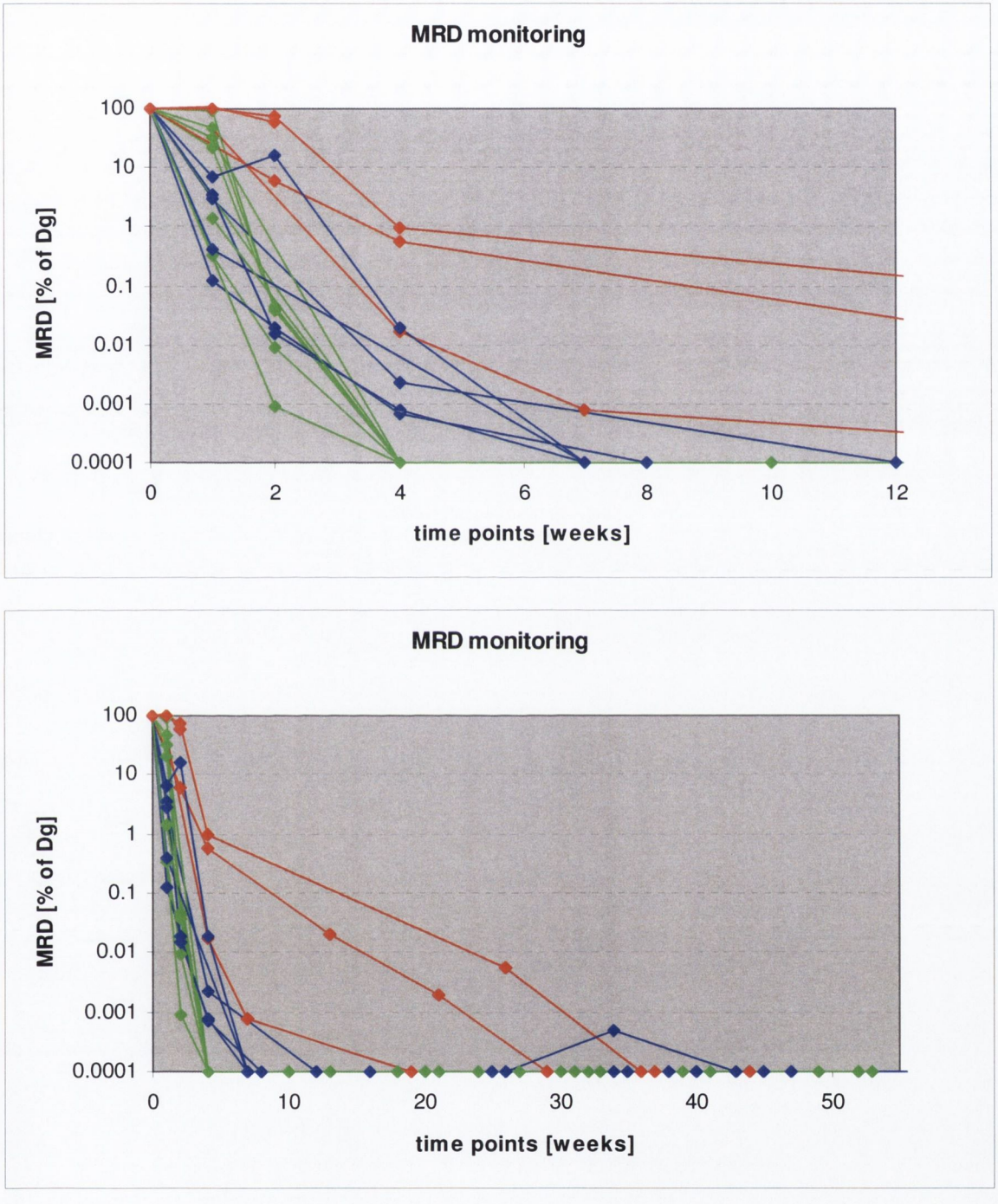


Figure 5.30. MRD monitoring in patients with childhood ALL during the first 12 weeks (upper panel) and the whole 1 year period (lower panel). Patients who achieved MRD negativity by day 29 are indicated in green, patients who achieved MRD negativity by the end of week 12 are indicated in blue, and patients who achieved MRD negativity late, after week 12, are indicated in red.

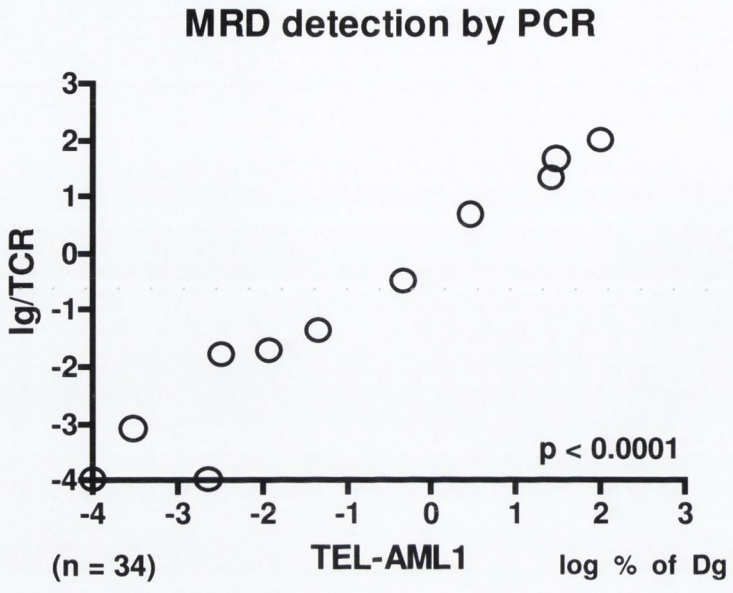


Figure 5.31. Correlation of results of MRD detection by PCR of chromosomal aberrations and of clonal IG/TCR rearrangements.

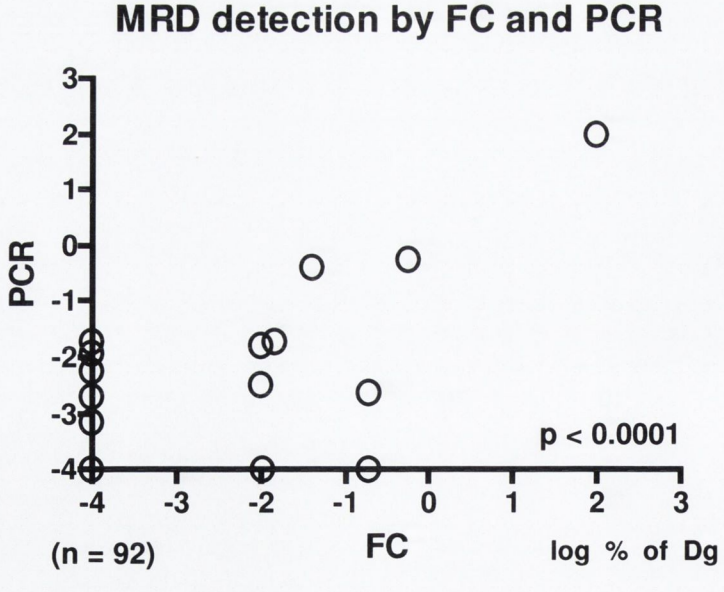


Figure 5.32. Correlation of results of MRD detection by FC and PCR methods.

5.10.2. Correlation of MRD levels with clinical data

According to the MRD presence in the most important time-points - at the end of induction and start of consolidation therapy on day 29 and week 12, respectively, the ALL patients could be divided into 3 groups: 1. patients MRD negative by day 29; 2. patients MRD negative by week 12; 3. patients MRD negative after week 12.

Of the 16 ALL patients, 7 patients (44 %) were MRD negative by day 29 and remained negative during the whole follow-up. Three of them were stratified into a risk group A (for the risk stratification see Table 4.3.), 4 patients to a risk group B at the diagnosis, one of these being switched to group C due to slow bone marrow response during the first weeks of treatment. This patient died of sepsis within the first year from diagnosis, in remission.

Five patients (31 %) achieved MRD negativity by week 12. One patient belonged to a risk group B at the diagnosis, all the others into a group A. With one exception (an isolated MRD positivity in a very low level, reaching the sensitivity and specificity threshold, on week 34), all these patients continued to be MRD negative and stayed in remission.

Three patients (19 %) became MRD negative only after week 12. They were stratified into risk groups A, B and C, respectively, and the patient from the group B was switched to C due to slow initial bone marrow response. Once they achieved MRD negativity, they remained negative in further samples and stayed in remission during the follow-up.

One patient (6 %) from the risk group B with MRD positivity on day 29 died of sepsis before the next follow-up.

Of the 2 AML patients, one with relapsed leukaemia showed very high levels of MRD in a sample from the week 7 – 12 and died of the relapse soon afterward. The other patient had a medium MRD level in a sample from this time-point and was indicated for bone marrow transplantation. No more samples were received from this patient.

No leukaemia relapse was diagnosed in the patients during the follow-up period (Table 5.14.).

Table 5.14. Clinical characteristics of patients with different MRD response.

Clinical characteristics of patients with different MRD response						
ALL patients						
UPN	Dg	Age	Chr. aberr.	WBC ≥ 50	Risk group	Outcome
MRD negative by day 29						
1	B-ALL	3	TEL-AML1	no	A	remission
3	B-ALL	11		no	B → C	died of sepsis
4	B-ALL	4	TEL-AML1	yes	B	remission
6	B-ALL	3		no	A	remission
14	B-ALL	5		no	A	remission
18	B-ALL	12		no	B	remission
22	T-ALL	3		yes	B	remission
MRD negative by week 12						
5	B-ALL	5	TEL-AML1	yes	B	remission
10	B-ALL	5	TEL-AML1	no	A	remission
13	B-ALL	4	TEL-AML1	no	A	remission
16	B-ALL	4	TEL-AML1	no	A	remission
17	B-ALL	4		no	A	remission
MRD negative after week 12						
2	B-ALL	1	TEL-AML1	no	A	remission
7	B-ALL	4	hypodiploidy	no	C	remission
12	B-ALL	6		yes	B → C	remission
Follow up finished after day 29						
9	B-ALL	14	(MLL?)	yes	B	died of sepsis
AML patients						
UPN	Dg	Age	Chr. aberr.	WBC ≥ 50	Risk group	Outcome
MRD positive on week 7 - 12						
24	AML	1				after BMT
25	AML	4				died of relapse

(UPN – unique patient's number, Dg – diagnosis, Chr. aberr. – chromosomal aberrations)

5.11. RQ-PCR detection and quantification of WT1 expression in childhood acute leukaemia and its use for MRD monitoring

5.11.1. WT1 expression in control haematopoietic tissues for assessment of normal ranges of WT1 expression

WT1 gene expression presence and levels were examined in control samples (normal peripheral blood - PB, normal and regenerating bone marrow - BM, peripheral stem cells - PSC) to assess normal ranges of *WT1* expression in these tissues as a basis for evaluation of *WT1* expression in leukaemia samples.

RT-PCR of total *WT1* gave weak positivity for normal BM and was negative for normal PB in first round reaction; nested reaction was positive for both normal BM and PB (Figure 5.33.).

In RT-PCR of *WT1* isoforms, normal BM was weakly positive for variants B and D (corresponding with the slightly higher sensitivity of these reactions) and negative for variants A and C by first round PCR; normal PB was negative for all *WT1* variants. With nested PCR, normal BM was positive for all four variants, normal PB (only one sample tested) was positive only for variants C and D (containing KTS fragment) (Figure 5.34.).

With RQ-PCR of total *WT1*, *WT1* expression could be detected in all control samples. Normal BM showed quite uniform pattern of *WT1* expression, with WT1 NCN within 1 log (median 29; range 22 – 115 x 10^{-4} WT1/ABL NCN). *WT1* expression in normal PB was lower and slightly more variable (2; 0.2 – 13). The only one PSC sample examined expressed *WT1* in a level analogous to normal PB. MRD-negative regenerating bone marrow taken at different time-points during the course of anti-leukaemic therapy exhibited median *WT1* expression almost identical to that of normal BM (median 28; range 1 – 100 x 10^{-4} WT1/ABL NCN). The range of detected *WT1* levels was wider in comparison with normal BM due to lower and more variable *WT1* expression in early follow-up samples (day 29 – week 16). In later follow-up samples (month 6 and 12), *WT1* expression was quite comparable to normal BM (Table 5.15. and Figure 5.35.).

The quantification of *WT1* expression in normal samples using the other control gene, *B2M* (WT1/100 B2M NCN), gave a very good correlation of results for bone marrow (median 25; range 18 – 52 x 10^{-4} WT1/100 B2M NCN). In peripheral blood, *WT1* levels calculated to *B2M* were slightly lower (0.7; 0.1 – 1) than when calculated to *ABL*, and so was the *WT1* expression in PSC (1 x 10^{-4} WT1/100 B2M). However, this difference never reached any statistical significance (Table 5.15. and Figure 5.36.).

Based on the examined control samples and allowing for eventual further variations in physiologic *WT1* expression, normal ranges of *WT1* expression in BM were defined as 75 % of the minimal and 125 % of the maximal *WT1* expression observed in normal BM (15 – 150 x 10^{-4} WT1/CG NCN).

RQ-PCR detection of *WT1* isoforms, exon 5-positive and KTS-positive, showed a different expression pattern in normal bone marrow and normal peripheral blood samples. In normal bone marrow, the expression of *WT1-EX5* was slightly lower (median 16; range 8 – 33 x 10^{-4} WT1/100 CG NCN) and *WT1-KTS* slightly, not significantly, higher (median 81; range 34 – 226 x 10^{-4} WT1/100 CG NCN) than the total *WT1*. The ratio *WT1-KTS/WT1-EX5* was around 3 (median 3.7; range 2.6 – 12). In normal peripheral blood, *WT1-EX5* expression was again very close to the total *WT1* (median 1; range 0.3 – 4 x 10^{-4}

WT1/100 CG NCN) while *WT1-KTS* isoforms showed, quite unexpectedly, much higher levels of expression than total *WT1* (median 40; range 16 – 552 x 10^{-4} WT1/100 CG NCN), with the ratio *WT1-KTS/WT1-EX5* around 80 (median 81; range 15 – 502). In the only peripheral stem cell sample, *WT1-EX5*, *WT1-KTS* and their ratio were 1, 5 (x 10^{-4} WT1/100 CG NCN) and 5, respectively (Table 5.16. and Figure 5.37.).

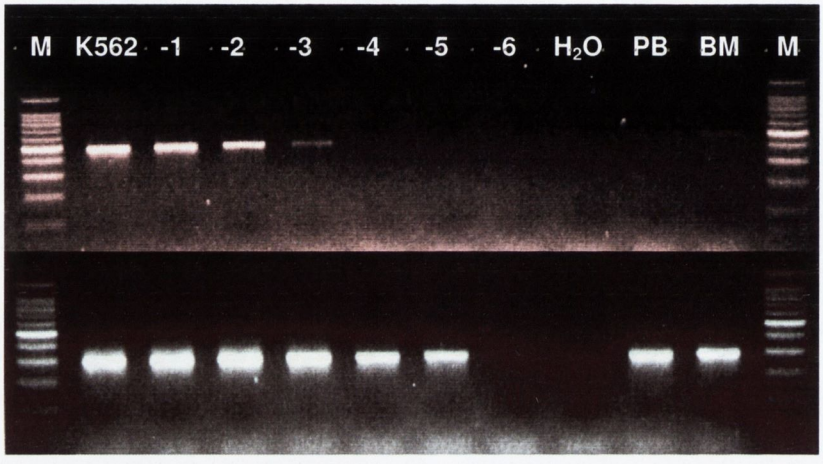


Figure 5.33. RT-PCR for total WT1, first round (upper panel) and nested (lower panel). Expression of WT1 in normal PB and BM, in comparison with K562 cell line dilution series (M – size marker).

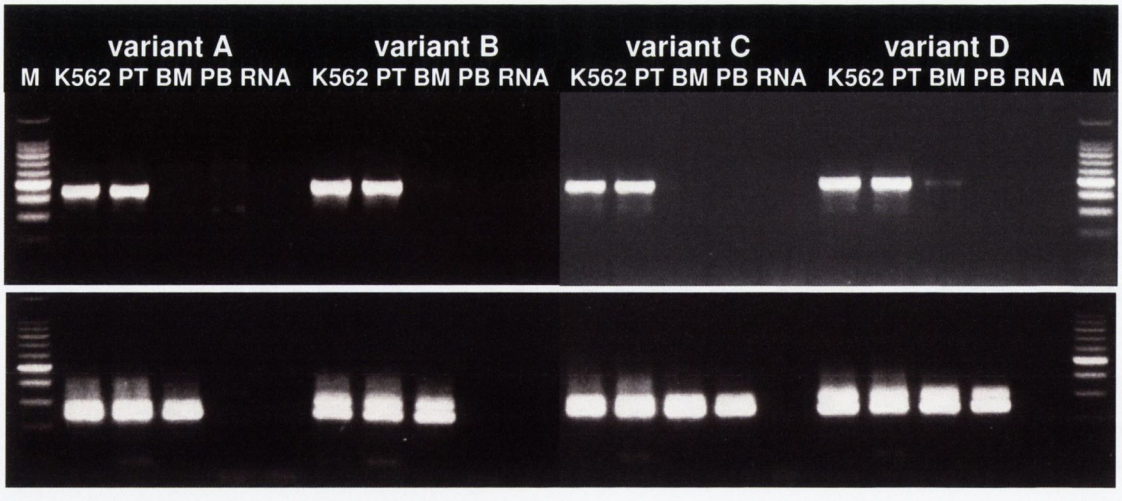


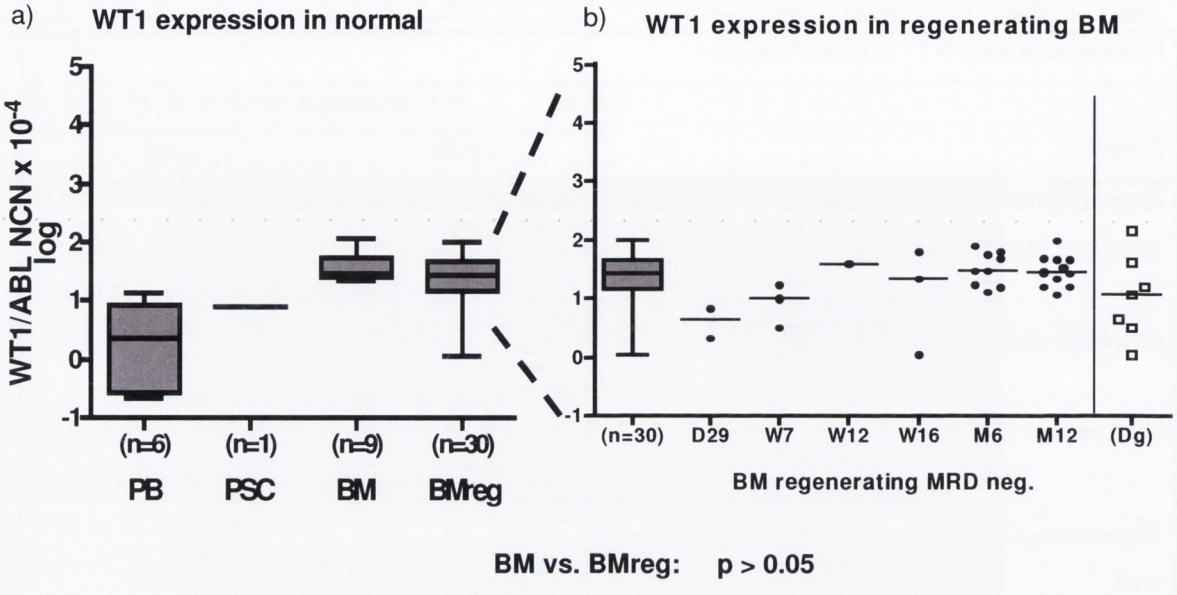
Figure 5.34. RT-PCR for WT1 isoforms, first round (upper panel) and nested (lower panel). Detection of WT1 isoforms in K562 cell line, acute leukaemia patient's sample, normal BM and PB samples (M – size marker, RNA – E. coli rRNA, a negative control).

Table 5.15. Expression of total WT1 in control samples.

Expression of total WT1 in control samples			
Samples	No	WT1 expression [NCN x 10^{-4}]	
		median	range
Normal donors (n = 16)			
peripheral blood (PB)	6	2 (WT1/ABL)	0.2 – 13 (WT1/ABL)
		0.7 (WT1/100 B2M)	*0.1 – 1 (WT1/100 B2M)*
peripheral stem cells (PSC)	1	8 (WT1/ABL)	
		1 (WT1/100 B2M)	
bone marrow (BM)	9	29 (WT1/ABL)	22 – 115 (WT1/ABL)
		25 (WT1/100 B2M)	*18 – 52 (WT1/100 B2M)*
Regenerating BM (MRD-) (n = 30)			
total	30	28	1 – 100
D29	2	4	2 – 7
W7	3	10	3 – 18
W12	1	39	
W16	3	22	1 - 63
M6	9	31	13 – 81
M12	12	29	12 - 100

Table 5.16. Expression of WT1 isoforms in control samples.

Expression of WT1 isoforms in control samples					
Samples	No	WT1-EX5 expression [NCN x 10^{-4}]		WT1-KTS expression [NCN x 10^{-4}]	
		median	range	median	range
Normal donors (n = 16)					
peripheral blood (PB)	6	1	0.3 – 4	40	16 – 552
peripheral stem cells (PSC)	1	1		5	
bone marrow (BM)	9	16	8 - 33	81	34 - 226



Figurte 5.35. WT1 expression in control samples: WT1 expression in normal donors and regenerating MRD-neg. bone marrow (BM) (a); W1 expression in regenerating MRD-negative BM from different time-points (and for comparison, WT1 expression of these patients at diagnosis) (b).

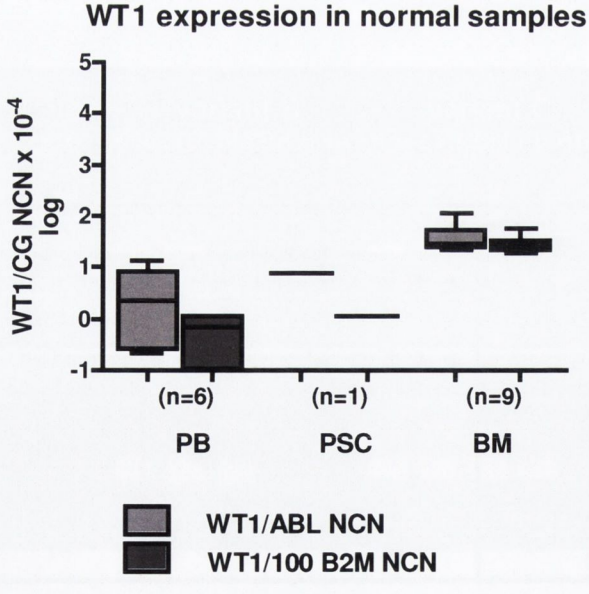


Figure 5.36. Expression of total WT1 in normal control samples related to the expression of two control genes, ABL and B2M.

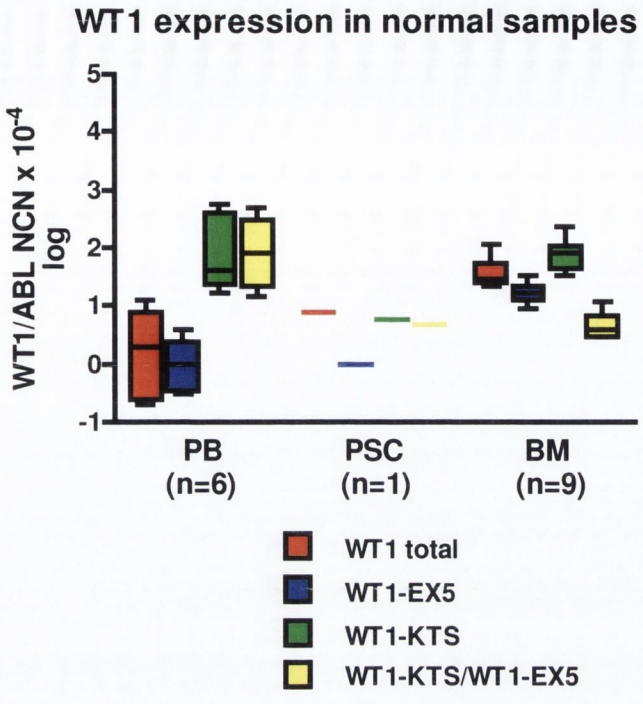


Figure 5.37. Expression of WT1 isoforms in normal control samples.

5.11.2. WT1 expression in diagnostic samples of childhood acute leukaemia patients, evaluation of WT1 as a potential MRD marker and independent prognostic factor

WT1 expression in childhood acute leukaemia

RT-PCR of total *WT1* and its isoforms were performed initially on a limited group of patients' samples. With RT-PCR for total *WT1*, all acute leukaemia patients were positive by the first round reaction, although with variable intensity of the bands, and all were strongly positive by nested PCR (Figure 5.38.). The samples with stronger total *WT1* expression were also positive for all *WT1* variants by first round as well as nested RT-PCR for *WT1* isoforms (Figure 5.34.).

By RQ-PCR, expression of total *WT1* in diagnostic bone marrow samples of childhood B-ALL varied within a wide range, over 5 logs (median 24.5; range 0 – 11 197 x 10^{-4} WT1/CG NCN). However, the majority of patients had *WT1* levels close to those of normal bone marrow and there was no significant difference between median *WT1* expression in B-ALL and normal BM ($p > 0.05$). Apart from several patients with considerable *WT1* overexpression, there was also a group of patients (almost one third) with very low *WT1* expression even below the minimal *WT1* levels in normal BM, including 2 patients in whom *WT1* was undetectable.

In contrast to B-ALL, *WT1* overexpression was found in most of childhood T-ALL cases (median 485; range 23 – 6 295 x 10^{-4} WT1/CG NCN). Though the difference in median *WT1* expression between

childhood T-ALL and normal BM did not reach the statistical significance ($p > 0.05$), *WT1* expression in childhood T-ALL was significantly higher than in B-ALL ($p < 0.001$).

Among all examined childhood ALL samples, even those with *WT1* overexpression, none expressed *WT1* in levels at least 2 log above the *WT1* expression detected in normal bone marrow (Table 5.17. and Figure 5.39.).

In paired peripheral blood and bone marrow samples of B-ALL patients, *WT1* expression in PB correlated well with *WT1* levels in BM but in slightly lower values (median 45; range 5 – 143 x 10^{-4} WT1/CG NCN) (Table 5.17. and Figure 5.39.).

AML patients showed variable *WT1* expression levels (median 475; range 28 – 922 x 10^{-4} WT1/CG NCN). In bone marrow samples of patients affected with other haematological malignancies (NHL, MDS), *WT1* expression was similar to that of normal BM (median 66; range 24 – 66 x 10^{-4} WT1/CG NCN) (Table 5.17. and Figure 5.39.).

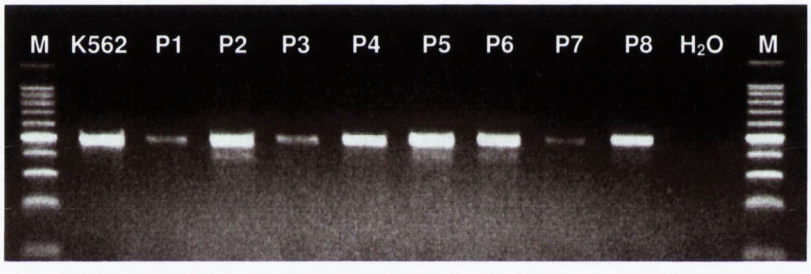


Figure 5.38. RT-PCR for total WT1, first round reaction: WT1 expression in acute leukaemia patients' samples (P1 – 8) (M – size marker, K562 – cell line, positive control).

Expression of *WT1* isoforms was evaluated in samples with a sufficient amount of RNA available. The expression of exon 5 isoforms was very similar or slightly lower than total *WT1* in all patients' subgroups – B-ALL (median 23; range 0 – 27219 x 10^{-4} WT1/CG NCN), T-ALL (473; 26 – 3842), AML (333; 17 – 647) and other haematological malignancies (88; 40 – 99). In contrast, *WT1-KTS* isoforms were expressed in significantly higher levels than total *WT1* in B-ALL patients (median 284; range 0.1 – 59307 x 10^{-4} WT1/CG NCN), and slightly higher levels in the other patients with T-ALL (1060; 23 – 1788), AML (567; 303 – 831) and other haematological malignancies (380; 90 – 381). The ratio *WT1-KTS/WT1-EX5* was in B-ALL around 10 with very wide ranges (median 10.1; range 0.4 – 924), around 2 in T-ALL (1.8; 0.6 – 11), in AML around 9 (9.3; 1.3 – 17) and in other haematological malignancies around 3 (3.8; 1 – 9.4). Expression of *WT1-KTS* was significantly higher in ALL patients than in normal bone marrow ($p < 0.01$) while there was no significant difference between *WT1-EX5* levels in ALL patients and normal BM (Table 5.18. and Figure 5.40.).

Table 5.17. Expression of total WT1 in childhood patients' samples.

Expression of total WT1 in childhood patients' samples			
Samples	No	WT1 expression [NCN x 10^{-4}]	
		median	range
Childhood acute leukaemia (n = 127)			
B-ALL	106	24.5	0 – 11197
B-ALL peripheral blood	3	45	5 – 143
T-ALL	19	485	23 – 6295
AML	2	475	28 – 922
Other haematological malignancies	3	66	24 – 66
B-ALL – aberrations (n = 116)			
TEL-AML1	22	21	1 – 1083
MLL-AF4	4	7302	4331 – 11197
BCR-ABL	11	159	1 – 3998
no/other	79	30	0 – 9551
ALL – age, WBC (n = 125)			
age 1 - 10	87	21	0 – 6295
age ≥10	35	380	1 – 11197
age ≥ 1	3	9006	5597 – 9322
WBC < 50	95	31	0.01 – 9551
WBC ≥ 50	30	148	0 – 6295
ALL – remission/relapse (n = 125)			
remission	111	35	0.01 – 9551
relapse/no response	14	13	0 – 11197

Table 5.18. Expression of WT1 isoforms in childhood patients' samples.

Expression of WT1 isoforms in childhood patients' samples					
Samples	No	WT1-EX5 expression [NCN x 10^{-4}]		WT1-KTS expression [NCN x 10^{-4}]	
		median	range	median	range
Acute leukaemia (n = 122)					
B-ALL	102	23	0 – 27219	284	0.1 – 59307
T-ALL	18	473	26 – 3842	1060	23 – 17887
AML	2	333	17 – 647	567	303 – 831
Other malignancies	3	88	40 – 99	380	90 – 381

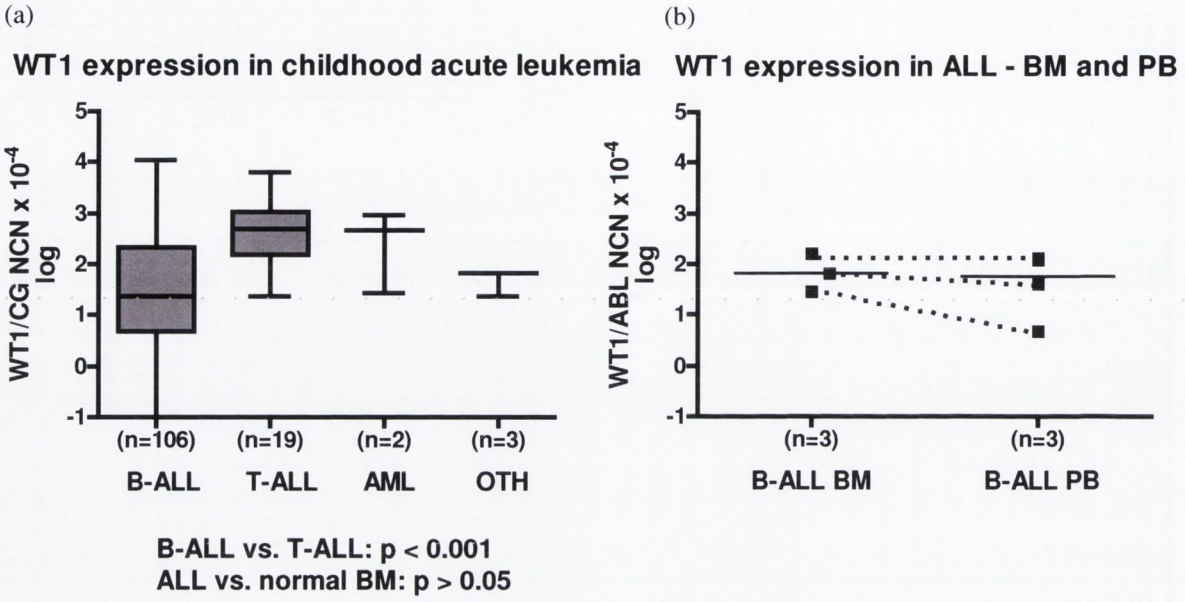


Figure 5.39. Expression of total WT1 in acute leukemia patients; in their diagnostic bone marrow (a) and paired B-ALL peripheral blood and bone marrow samples (b).

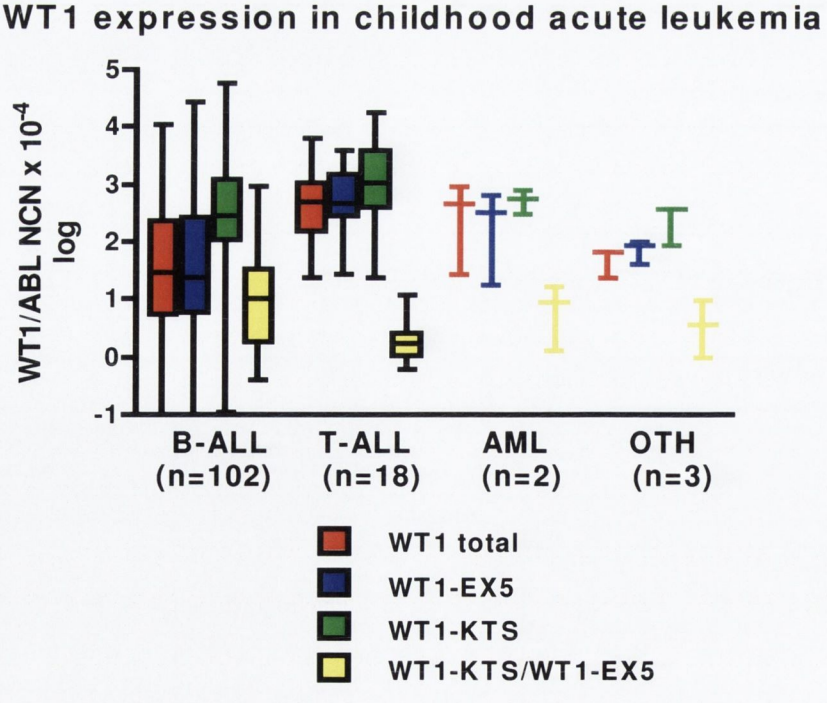


Figure 5.40. Expression of WT1 isoforms in childhood acute leukaemia.

WT1 expression in childhood ALL with different chromosomal aberrations

Total *WT1* expression was analyzed in subgroups of childhood B-ALL with following chromosomal aberrations: *TEL-AML1* (*ETV6-RUNX1*) translocation (the most frequent and prognostically rather favourable), *MLL-AF4* and *BCR-ABL* translocations (both prognostically unfavourable), and no/other aberrations. Patients with *MLL-AF4* translocation expressed remarkably high *WT1* levels (median 7302; range 4331 – 11 197 x 10^{-4} WT1/CG NCN) in comparison with all the other subgroups ($p < 0.01$), while there was no significant difference in *WT1* expression between patients with *TEL-AML1*, *BCR-ABL* and no/other aberrations ($p > 0.05$) (Table 5.17. and Figure 5.41.).

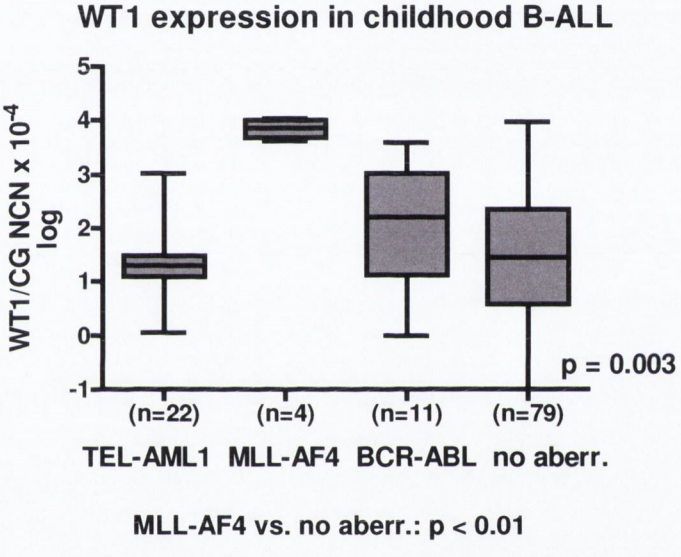


Figure 5.41. Expression of total WT1 in B-ALL patients with different chromosomal aberrations.

WT1 expression in childhood ALL and main risk factors – age, WBC

The relation between age and leukocyte count at diagnosis (the two principle risk factors in childhood ALL) and *WT1* expression was studied. Children at the age ≥ 10 years had *WT1* expression significantly higher (median 380; range 1 – 11 197 x 10^{-4} WT1/CG NCN) than children between 1 and 10 years of age (21; 0 – 6 295) ($p < 0.001$). Also infants ≤ 1 year showed high *WT1* expression (9006; 5 597 – 9 322) with the difference statistically significant ($p < 0.01$), but this finding is influenced by the fact that 2 of the 3 patients also had MLL-AF4 translocation.

In patients with peripheral blood WBC < 50 x 10^9 / l and leukocytosis ≥ 50 x 10^9 / l, no major difference in *WT1* expression was observed ($p > 0.05$) (Table 5.17. and Figure 5.42.).

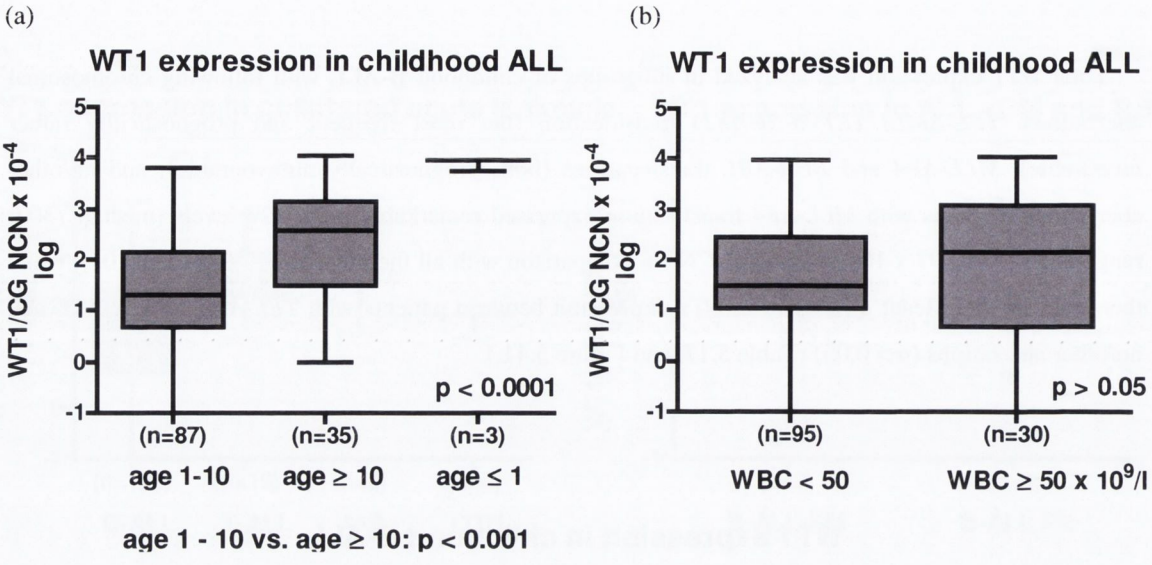


Figure 5.42. Expression of total WT1 in ALL patients according to risk factors – age (a) and peripheral white blood cell count (b) at diagnosis.

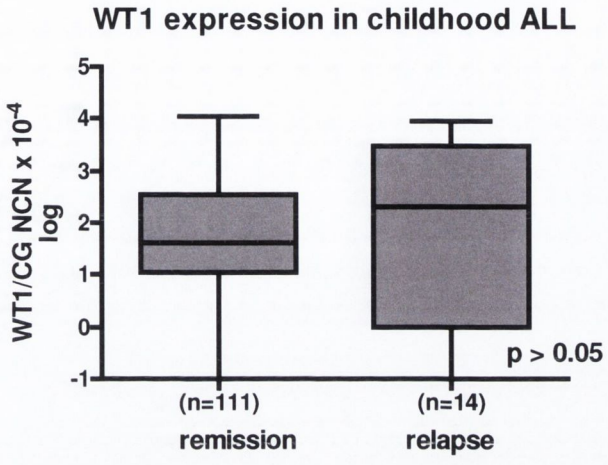
WT1 expression in childhood ALL and the risk of relapse

The 14 patients who have relapsed (including 1 with no response) did not differ in the median or range of *WT1* expression (median 13; range 0 – 11 197 x 10^{-4} WT1/CG NCN) from to the rest of patients staying in remission ($p > 0.05$). However, if all childhood ALL patients were divided into three groups with abnormally low (< 15), normal (15 – 150) or abnormally high (> 150) *WT1* expression (see definition of normal BM *WT1* expression previously), a significant difference was observed in relapse-free survival between these groups ($p = 0.012$; for low vs. normal *WT1* $p = 0.024$, for high vs. normal *WT1* $p = 0.015$). Patients with both low and high *WT1* expression had an increased risk of relapse with hazard ratio 11.6 (95% CI 1.4 – 97) and 13.5 (1.7 – 111), respectively. In multivariate analysis including age and WBC (the presence of unfavourable chromosomal aberrations *BCR-ABL* and *MLL-AF4* was not included because of too few cases in the cohort of patients; both 2 *BCR-ABL* positive patients and none of the 3 patients with *MLL-AF4* relapsed), *WT1* under- and overexpression proved to be an independent risk factor of relapse ($p = 0.014$ and 0.049, respectively). It was more pronounced for the patients with abnormally low *WT1* expression, while in patients with abnormally high *WT1* levels the relapse was often associated with the age over 10 years. However, this may reflect the fact that in the whole group of patients over 10 years of age higher *WT1* levels were detected.

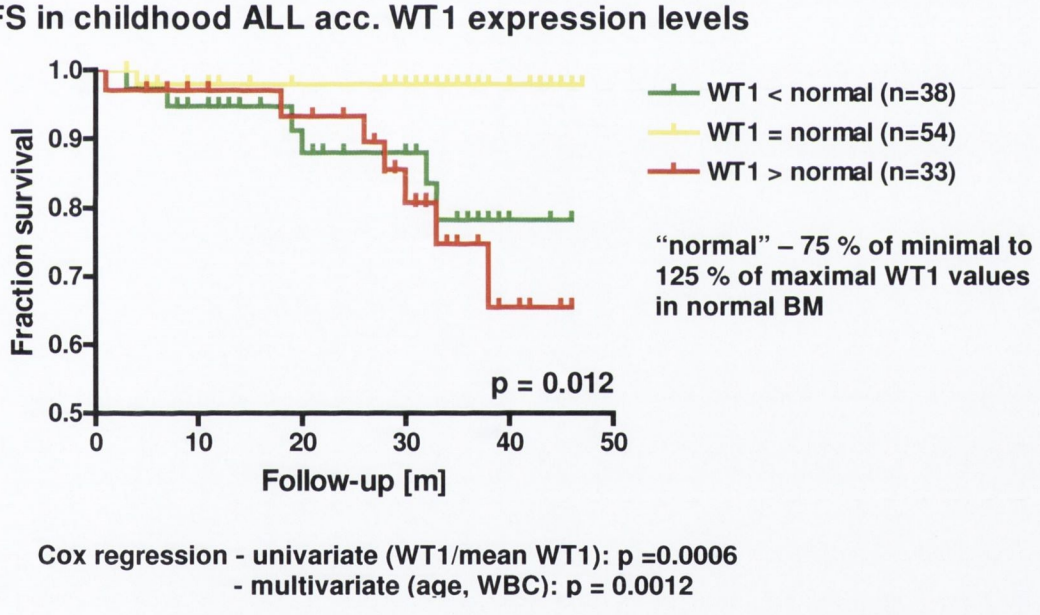
Similar, even clearer results were obtained when univariate and multivariate analysis were performed with the individual values of *WT1* expression (their absolute difference from the mean *WT1* expression in ALL). In univariate analysis, *WT1* levels increasing or decreasing from the mean *WT1* expression were associated with significantly increased risk of relapse ($p = 0.0006$) with hazard ratio 1.5 (1.2 – 1.9) / 2-

fold increase or decrease; and the independence of *WT1* prognostic value was confirmed in multivariate analysis (p = 0.0012) (Table 5.17. and Figure 5.43.).

(a)



(b)



Cox regression - univariate (WT1/mean WT1): p =0.0006
- multivariate (age, WBC): p = 0.0012

Figure 5.43. Expression of total WT1 in ALL patients and the risk of relapse; WT1 expression in patients in remission and relapsed (a), relapse-free survival in patients with different levels of WT1 expression (b).

Correlation of WT1 and CD34 expression in childhood ALL

To determine a potential relation between *WT1* levels and CD34 expression in childhood ALL, *WT1* expression and percentage of CD34 positive leukemic cells at diagnosis were compared (the data on CD34 expression were available for 126 of 136 samples). No correlation was found (p > 0.05).

Detection of MRD in childhood ALL using WT1 overexpression

In acute leukaemia patients with detected high overexpression of *WT1* at diagnosis, *WT1* was evaluated as a potential marker for MRD monitoring in the follow-up samples. Considering the minimum sensitivity and specificity required from assays for MRD detection, the threshold for *WT1* overexpression in bone marrow diagnostic samples suitable for further MRD monitoring was set to WT1 NCN 10 000 x 10^{-4} copies of WT1/ABL, to be 2 log higher than the upper limits of *WT1* expression in normal bone marrow. None of the samples from the MRD part of this study and only 1 sample from the whole WT1 cohort (with the presence of *MLL-AF4* translocation) fulfilled these criteria. No MRD monitoring by *WT1* expression was therefore performed and *WT1* does not appear to be a suitable marker for MRD detection in childhood ALL.

6. Discussion

6.1. Detection of minimal residual disease in childhood acute leukaemia by flow cytometry

6.1.1. Methodology

The method of detection of MRD in childhood acute leukaemia by four-colour flow cytometry has been established following general recommendations for immunophenotyping of acute leukaemia by flow cytometry and suggested operating procedures for flow cytometry with regards to the quality assurance [Ormerod *et al* 2000, Bain *et al* 2002, Lucio *et al* 1999 and 2001, Porwit-MacDonald *et al* 2000, Campana *et al* 1999, 2000, 2001 and 2002, Owens *et al* 2000], according to the latest knowledge and available information on MRD detection in leukaemia by flow cytometry [Ciudad *et al* 1998, Tiensiwakul *et al* 1999, Orfao *et al* 1999, Griesinger *et al* 1999, Nagler *et al* 1999, Bene *et al* 1999 and 2001, Legrand *et al* 2000, van Lochem *et al* 2000, van Wering *et al* 2000, Oelschlagel *et al* 2000, Venditti *et al* 2000, Coustan-Smith *et al* 2000 and 2002b, San Miguel *et al* 2001 and 2002, Baer *et al* 2001, McKenna *et al* 2001, Deneys *et al* 2001, Scolnik *et al* 2002, Rizzatti *et al* 2002, Dworzak *et al* 1997 and 2002, Reinhardt *et al* 2002, Sanchez *et al* 2002], with the use of our previous experience in three-colour flow cytometric MRD detection in childhood ALL, and based on the results of careful and precise method optimisation. Studies published later, during the time of optimisation and routine use of FC for MRD detection, have entirely supported our chosen approach [Campana *et al* 2003 and 2004, Veltroni *et al* 2003, Borowitz *et al* 2003, Bjorklund *et al* 2003, Vidriales *et al* 2003, Coustan-Smith *et al* 2003, Kern *et al* 2003, Sievers *et al* 2003]. The characteristics of the method and its parameters, namely applicability, reproducible sensitivity and specificity, fulfil the criteria required for reliable MRD detection and monitoring by flow cytometry.

Sample handling

A proper sample collection and handling is an essential prerequisite for reliable FC analysis and MRD detection, as confirmed by our own results as well as conclusions of most of the recent publications on MRD detection in leukaemia. Careful collection of a bone marrow sample without peripheral blood contamination and damaging the surrounding tissues, use of appropriate anticoagulant solutions (K_2 or K_3EDTA, ACD, preservative-free heparin), quick and gentle transport of the sample to the lab at room temperature, and its immediate processing (within 24 hours, ideally within 4 – 8 hours) prevent cell damage and loss, changes in antigen expression patterns, and thus reduce the risk of inaccurate results. A collection of a defined amount of bone marrow sample allows an absolute MRD quantification per sample volume which may have additional prognostic implications [Dworzak *et al* 2002].

Choice of fluorochromes for four-colour flow cytometry

Four-colour flow cytometry, enabled by the development of dual-laser flow cytometers, has several advantages over the original three-colour method. By simultaneous detection of six independent FC parameters (FSC, SCC and 4 different fluorochromes marking 4 independent antigens) it may not only reduce the amount of cells required for the experiment but, most importantly, it allows better definition of the cell subsets using multiple gating strategy and evaluation of multiple antigen co-expression. New types of fluorochrome dyes, that have become recently available, may further increase the opportunity of simultaneous detection of different antigens to 5 – 8, and offer additional improvement in immunophenotyping of acute leukaemia and MRD detection by flow cytometry in the nearest future.

The choice of fluorochromes employed for the 4-colour FC MRD detection may be a matter of individual experience and preferences, and should respect the particular objectives. The most widely and standardly used fluorochromes are FITC for the first channel, PE for the second channel and APC for the fourth channel. For the third channel, 2 fluorochromes, PerCP or PECy5 are commonly used (PerCP more often). Each of them shows some advantages and disadvantages: PerCP has generally lower intensity of fluorescence which may lead to false negative results in detection of weakly expressed antigens; PECy5 gives on the contrary very high levels of fluorescence intensity requiring a careful titration, as a conjugate of two fluorochromes it is also less stable and requires careful handling, protection from light and storage at low temperatures (4 °C) all the time to prevent high unspecific background and false positive results. Also, more antibodies are available as PECy5 conjugates than PerCP conjugates. For the purpose of MRD detection in acute leukaemia we decided to use PECy5 fluorochrome to be able to reliably detect even weakly expressed antigens and to have an option of more antibody combinations. However, because of the risk of possible unspecific positivity we would not like to use PECy5-labelled antibody on its own for definition of LAPs but only in combination with other antibodies and especially for gating purposes.

Identification of LAPs

For the identification of LAPs in the diagnostic acute leukaemia samples, we have designed and used extensive panels of antibody combinations that allowed detection of practically all known LAPs. The main aim of this approach was to address the question of benefit of both most common and rare LAPs for MRD detection in childhood acute leukaemia. The results of clinical samples analysis showed that only a limited group of LAPs types were found in the tested samples; these LAPs enabled reliable MRD definition and monitoring in all samples. This finding is in agreement with results of other published studies [Lucio *et al* 2001, Coustan-Smith *et al* 2000, 2002b and 2003, Dworzak *et al* 2002, Rizzatti *et al* 2002, Borowitz *et al* 2003, Sievers *et al* 2003, Veltroni *et al* 2003] where authors describe MRD detection in acute leukaemia samples with a limited set of antibody combinations. Based on ours as well as other authors' findings we would suggest a two-step approach for further studies on detection of LAPs in acute leukaemia for MRD monitoring purpose: (1) testing all diagnostic samples with first-line antibody panels that would contain up to 5 antibody combinations detecting the most frequent LAPs in the particular leukaemia type (B-ALL, T-ALL, AML); (2) using second-line antibody panels containing further 5 – 10 antibody combinations detecting other LAPs, only in samples where less than 2 reliable LAPs could be identified with the first line panel. This approach will considerably reduce the costs and time of FC MRD

detection without affecting its accuracy and would be suitable for standard MRD detection in routine laboratory practice. In a routine haematology laboratory setting, the first-line panel detection of LAPs could also be combined or included in the antibody panel used for leukaemia immunophenotyping at diagnosis.

Staining for FC

The optimised protocols of sample staining for FC analysis must be strictly followed to obtain the best results. The quality of the staining procedure may in the future be further improved by updating the protocols as a response to newly developed reagents and techniques. It is generally known and we have repeatedly noticed that antibodies of other isotypes than IgG1 (IgG2a and b, IgM) show significantly higher unspecific binding which may thus decrease the sensitivity of MRD detection. It would be therefore worth replacing these antibodies with IgG1 isotypes once they are available. During the optimising phase we have replaced CD7-FITC antibody of IgG2b isotype (DAKO) with CD7-FITC antibody of IgG1 isotype (BD) that led to improved detection of MRD in AML with cross-lineage expression of CD7. Recently, FITC-labelled CD15 and CD65 antibodies of IgG1 isotype can be purchased (Cytognos) that could have similar benefit in detection of LAPs involving CD15 and CD65 antigens. Any changes in the protocols have to undergo the necessary optimisation steps (e.g. titration of the new antibodies) before being approved for standard use.

The method of whole blood lysis instead of mononuclear separation on a gradient medium seems to be quite acceptable for FC MRD detection, as proven by our own work and a number of other authors [Venditti *et al* 2000, San Miguel *et al* 2001, Dworzak *et al* 2002, Borowitz *et al* 2003, Bjorklund *et al* 2003, Vidriales *et al* 2003, Veltroni *et al* 2003]. This method makes the preparation of the sample for FC analysis faster, may decrease antibody unspecific binding (due to the blocking effect of present plasma) and exclude residual contaminating erythrocytes and debris from the analysis. Some other authors [Campana *et al* 2002] argue against this method suggesting that granulocytes and platelets remaining after erythrocyte lysis may increase background staining and dilute leukaemic population. However, in our hands the use of erythrocyte lysing technique notably reduced the unspecific background and improved MRD quantification, without decreasing its sensitivity.

Sample acquisition and analysis

The samples acquisition and analysis were carried out in a standardised way on the flow cytometer. Regular maintenance and calibration of the instrument, use of appropriate controls and regular checking of all parameters are important for quality assurance and generation of valid data. We were using individual patients' compensation parameters settings, which is an approach that is seldom referred by other authors. The purpose of this technique is to compensate for the individual variations in cell autofluorescence. We could demonstrate that this approach may improve partial spectral overlap present when common compensation parameters are used, and potentially leading to false positive results. Individual parameter setting thus may increase specificity and sensitivity of the method and decrease a risk of incorrect results in some patients.

As the sensitivity of the method depends on the total number of events analysed, an acquisition of a sufficient amount of cells is crucial for MRD detection. We managed to analyse over 500 000 events per 1 antibody combination in vast majority of the examined samples, allowing sensitivity of 10^{-4} – 10^{-5} to be realistically achieved (see below).

The multiple gating strategy including the "live" gating has proven to be a helpful step in FC MRD detection. It improves the discrimination of leukaemic population and may considerably diminish the unspecific background, thus again increasing the specificity and sensitivity of the method. This technique has been used already in several other studies on MRD detection published recently [San Miguel *et al* 2001, Vidriales *et al* 2003, Bjorklund *et al* 2003, Borowitz *et al* 2003, Sievers *et al* 2003].

Applicability

Detection of MRD by flow cytometry can be performed in about 90 % of acute leukaemia cases [Campana *et al* 2003]. We were able to define suitable LAPs and use them successfully for MRD monitoring in all analysed patients.

Specificity

The specificity of the individual LAPs was defined according to their presence and levels in control samples. As control samples we used normal adult donors' bone marrow samples (no normal childhood bone marrow samples were available to us), bone marrow samples from childhood acute leukaemia patients not expressing the tested LAPs and with a different type of leukaemia, and follow-up bone marrow samples of patients with confirmed MRD negativity by PCR methods. It is well known that antigen expression profile of normal bone marrow from adults and children may differ, and also that the antigen expression in bone marrow during and after chemotherapy changes significantly from that of normal bone marrow [Campana *et al* 2002 and 2003, Dworzak *et al* 1997, 2002 and 2003, Coustan-Smith *et al* 2000 and 2002b, van Lochem *et al* 2000, van Wering *et al* 2000, Gaipa *et al* 2005]. We thus consider including samples from childhood donors and from patients regenerating after chemotherapy among controls as absolutely necessary for correct definition of LAPs and quantification their background level of expression in normal samples.

We could analyse the expression of all LAPs in what is admittedly a limited group of control samples. Although we obtained quite consistent findings enabling good definition of the regions where none or very low numbers (< 0.1 – 0.01 %) of cells in the control samples may be present, a substantially higher number of control samples, both normal donors and patient's samples at different time-points of therapy, should be tested to better delineate the ranges of individual variations in antigen expression. The frequencies, in which normal and regenerating cells may occur in LAPs defining regions of acquisition plots, could be then more accurately calculated by statistical methods [Campana *et al* 2002, Kern *et al* 2003].

Sensitivity

The achieved sensitivity of the method itself is high. The reproducible sensitivity of detection of particular LAPs is determined by the LAPs specificity and by the number of total cells acquired, ranging between 10^{-3} – 10^{-5} cells.

In ALL patients, at least 1 LAP with sensitivity of 10^{-4} or higher was found in all but one (T-ALL) patients. In both AML patients, all the LAPs identified showed lower sensitivity of 10^{-3}. This is in agreement with published data also finding this lower sensitivity of MRD detection in AML, especially in childhood AML [Reinhardt *et al* 2002, San Miguel *et al* 2002, Coustan-Smith *et al* 2003, Kern *et al* 2003]. It is probably caused by the different characteristics of the most common LAPs in AML (mainly asynchronous antigen expression), and by the heterogeneity of AML leukaemic population. Contrary to ALL, AML cells on FC analysis do not form a dense cluster of cells, being spread over much larger regions, and even multiple gating cannot separate leukaemic from normal cells as clearly as it does in case of ALL. Also, in comparison with ALL where a particular LAP is expressed by the vast majority of cells or whole leukaemic population, in AML even the most frequent LAPs are expressed only by a fraction of leukaemic cells.

Overall characteristics of the method

Flow cytometry enables MDR detection in the vast majority of patients with acute leukaemia of all types and subtypes. In comparison with other methods of MRD detection, FC is the least standardised method and requires initial assessment of a substantial group of control samples. However, once established and optimised, it can produce results very quickly, within few hours, offers absolute and accurate quantification of MRD levels, and can distinguish between viable and dead leukaemic cells. It achieves good sensitivity and specificity of MRD detection, which is however slightly higher for ALL ($\geq 10^{-4}$) than AML ($\geq 10^{-3}$). On the other hand, for many AML patients (those without chromosomal aberrations or *IG/TCR* rearrangements) it represents the only available method of MRD monitoring. As the MRD markers (LAPs) may undergo changes during the disease course, more than one of them should be used for reliable MRD monitoring by FC.

Future prospectives

Present flow cytometers equipped with 2 – 3 lasers and a number of different fluorochromes available on the market enable simultaneous detection of 6 – 8 or even more different antigens at the same time and therefore a real multi-colour flow cytometry analysis. This decreases the amount of the patient's sample necessary for the analysis; the multiple antigen combinations also enable better definition of the leukaemic population and thus increase the specificity of the method. Novel fluorochrome dyes show superior characteristics over some of the older ones, most importantly a good stability of the conjugates and a bright signal, which may improve the discrimination of weakly expressed antigens and reduce the unspecific background.

The latest studies suggest that a concise panel of antibody combinations should enable identification of LAPs in the vast majority of patients [Dworzak *et al* 2002 and 2003, Veltroni *et al* 2003]. In contrast to the earlier studies, the later studies reveal that combinations targeting quantitative derangements in

antigen expression (e.g. overexpression of CD10, 20, 34, 58 in B-ALL, underexpression of CD38, 45 in B- and CD3 in T-ALL, quantitative changes in expression of CD34, 117, 11b in AML) represent probably the most reliable and applicable LAPs. These can be frequently found in acute leukaemia patients and modern methods of analysis allow precise discrimination between the quantitative changes in their expression on leukaemic cells and normal bone marrow precursors. The use of a limited set of antibody combinations targeted against quantitative LAPs may be well standardized, is suitable for a routine practice and would lower the costs of MRD detection in comparison with the individually tailored antibody panels employing mostly qualitative markers. It also seems that, in many patients, these quantitative LAPs may be used for MRD detection even without previous knowledge of the initial diagnostic leukaemia immunophenotype, which would be a further advantage over PCR-based methods [Dworzak *et al* 2003].

For the evaluation of MRD status, absolute enumeration of MRD (number of leukaemic cells per µl of the sample) may show advantages over relative MRD quantification (percent of leukaemic cells from all cells analysed) and raise the methodological accuracy, as it excludes intra- and inter-patients variations in the amount of regenerating normal bone marrow cells present [Dworzak *et al* 2002 and 2003].

The type of the sample used for MRD evaluation and the most indicative time-points remain a matter of debate. While it is well known that MRD values in paired peripheral blood and bone marrow samples are highly concordant in T-ALL, whereas large differences may occur in B-cell precursor ALL [Coustan-Smith *et al* 2002a, van der Velden *et al* 2002a], MRD monitoring in peripheral blood of B-ALL patients may still have high prognostic significance [Coustan-Smith *et al* 2002a] and the use of peripheral blood together or instead of bone marrow samples may be considered. The time-points of samples collection and MRD analysis should be defined with the aim to avoid the peak time of marrow regeneration after chemotherapy that may cause a higher unspecific background and decrease the accuracy of the MRD assessment [Dworzak *et al* 2003, Gaipa *et al* 2005].

6.1.2. Results of clinical samples analysis

Identification of MRD markers in diagnostic samples

By flow cytometry we were able to identify suitable LAPs and monitor MRD in all patients examined.

In 14 cases of B-ALL, 2 – 3 (median 2) different LAPs could be found in every diagnostic sample and used for consequent MRD tracking. This ensures that the accuracy of FC MRD detection should not be affected by eventual antigen switch/clonal development. At least one LAP with sensitivity $\geq 10^{-4}$ was detected in all B-ALL samples allowing a threshold for MRD detection of 10^{-4} - 10^{-5} in all patients. A limited number of 8 different LAPs types was present and could be used for MRD detection in all B-ALL samples. Among them, the most useful combination was CD58/10/34/19, expressed in over 90 % of the samples. Although it is a quantitative marker, the combination of strong expression of CD58 together with high overexpression of CD10 and aberrant expression of CD34 is usually well distinguishable from normal expression pattern of these antigens in control bone marrows. The sensitivity of this LAP could achieve very good levels of 10^{-4} - 10^{-5} in the samples tested. CD58 was not commonly used in previously published studies on MRD detection in ALL and we included it into the diagnostic panel based on our

previous early experience with MRD detection. A recently published paper [Veltroni *et al* 2003] describing CD58 as a novel marker for MRD monitoring in B-ALL with exceptional applicability, stability and accuracy, confirmed entirely our own experience. The other most common LAPs identified in our group of patients have been well known and recommended by most of the previous studies as frequent and suitable MRD targets [Griesinger *et al* 1999, Campana *et al* 1999 and 2002, Lucio *et al* 2001, Coustan-Smith *et al* 2000 and 2002b, Dworzak *et al* 2002, Bjorklund *et al* 2003, Vidriales *et al* 2003]. The use of anti-CD66c (KORSA) antibody instead of common anti-CD66 antibody may further improve the discrimination of the CD66/10/34/19 combination [Dworzak *et al* 2003, Kalina *et al* 2005].

In 2 T-ALL cases analysed by FC, only a single LAP could be identified in each of the diagnostic samples. In one of the patients, the LAP was represented by the most typical combination in T-ALL, TdT/c3/7/3. This LAP is considered to be a quite stable, sensitive and reliable MRD marker in T-ALL and has been successfully used on its own for MRD detection in many studies [Campana *et al* 2002, Coustan-Smith *et al* 2000 and 2002b, Dworzak *et al* 2002, Bjorklund *et al* 2003, Vidriales *et al* 2003]. In the other patient, the leukaemic cells showed antigen expression pattern rather unusual for childhood T-ALL. The only LAP that could be defined in this patient was CD8–/4–/7/3 pattern having limited sensitivity of detection (10^{-3}). The clinical course of the disease in this patient was atypical, too, with progression not responding to induction therapy and early death before the day 29 (Dr. Aengus O'Marcaigh, Department of Haematology, OLHSC Crumlin, personal communication).

In AML patients (2 cases), higher number of LAPs (4) was detected in the diagnostic samples. The kinds of the LAPs were quite typical for AML, sensitivity of all the LAPs was around 10^{-3}. These findings correspond well with other authors' results [Campana *et al* 2002, Coustan-Smith *et al* 2003, Reinhardt *et al* 2002, Kern *et al* 2003, Venditti *et al* 2000, San Miguel *et al* 2001 and 2002].

Monitoring of MRD in follow-up samples

In the 14 ALL patients with available follow-up samples, MRD was undetectable by FC from day 29 onward in 8 (57 %) of them. Further 2 patients (14 %) with low or medium MRD levels on day 29 achieved MRD negativity by week 7 – 12. The remaining patients (21 %) reached the MRD-negative status only after week 12. Once MRD-negative, all the patients remained in the molecular remission during the study follow-up. Both AML patients showed significant levels of MRD in their week 7- 12 samples. These results again correspond with findings of other similar studies [Ciudad *et al* 1998, Farahat *et al* 1998, Griesinger *et al* 1999, Coustan-Smith *et al* 2000, 2002b and 2003, Dworzak *et al* 2002, Bjorklund *et al* 2003, Borowitz *et al* 2003, Venditti *et al* 2000, San Miguel *et al* 2001, Kern *et al* 2003, Sievers *et al* 2003]

Correlation of MRD presence and levels detected in the same patients by different LAPs

When results of MRD detection by 2 different LAPs were compared, an overall good correlation was found ($p < 0.0001$ in Spearman rank test). However, the correlation was less evident for low levels of MRD (below 0.1 %) than for higher MRD levels, and inferior to the correlation of results of PCR MRD detection by 2 different clonal rearrangements. This is probably due to the method characteristics with

slightly lower sensitivity of FC over PCR-based methods and the potential influence of intensive bone marrow regeneration on FC results.

6.2. Detection of minimal residual disease in childhood acute leukaemia by PCR of chromosomal aberrations

6.2.1. Methodology

RT-PCR and RQ-PCR for detection of chromosome aberrations and fusion gene transcripts for MRD monitoring were established according to the BIOMED-1 Concerted Action and Europe Against Cancer Program recommendations and protocols [van der Velden 2003 and 2004, van Dongen 1999, Gabert 2003, Beillard 2003] with respect to the latest development in this area [Gameiro *et al* 2002, Lo-Coco *et al* 2003a, Cazzaniga *et al* 2002b, Liu Yin *et al* 2002, Grimwade *et al* 2002] and based on our previous experience. All reactions were optimised and validated on groups of control samples. The parameters of the assays, their efficiency, specificity, sensitivity and reproducibility, fulfil the BIOMED-1 and EAC requirements for MRD detection by PCR of chromosomal aberrations.

RT reaction

Synthesis of cDNA was performed for the initial optimising experiments according to the suggested BIOMED-1 protocol [van Dongen *et al* 1999]. Later, it was slightly modified according to the new recommendations of EAC [Gabert *et al* 2003, Beillard *et al* 2003] using increased concentration of random hexamers and decreased amount of reverse transcriptase. The EAC protocol proved to be more efficient and improved the sensitivity of consequent PCR reactions by up to 1 log. This protocol was then used for cDNA synthesis for RT-PCR and RQ-PCR detection of chromosomal aberrations in all clinical samples.

RT-PCR

RT-PCR for chromosomal aberrations was set up according to the BIOMED-1 protocol [van Dongen *et al* 1999] and every reaction was optimised by titration of magnesium concentration. In addition, titration of optimal DMSO concentration was carried out for all PCR reactions for t(1;19) *E2A-PBX1* t(12;21), *TEL-AML1* and microdel (1p) *SIL-TAL1*. In case of PCR reactions for t(1;19) *E2A-PBX1* and t(12;21) *TEL-AML1*, addition of neither DMSO nor other enhancers of PCR reaction is recommended in the BIOMED-1 protocol. However, in our hands the PCR for t(1;19) *E2A-PBX1* was very inefficient and the PCR for t(12;21) *TEL-AML1* generated results with lower sensitivity than would be expected. We tested addition of DMSO and other enhancers (BSA, trehalose, glycerol) to improve the performance of the reactions. DMSO in low concentration gave the best results and was therefore used for all consequent experiments. For PCR of microdel (1p) *SIL-TAL1*, the BIOMED-1 protocol recommends supplement of 10 % DMSO. This seemed rather high and the results obtained were not of a good quality, so DMSO titration was performed again to improve the reaction outcome.

RQ-PCR

RQ-PCR for quantification of fusion gene transcripts from chromosomal aberrations was established according to the EAC protocols [Gabert *et al* 2003, Beillard *et al* 2003]. These protocols are already optimised and in our hands all of them performed very well, so no special further work up was necessary. The characteristics of the reactions were all within the parameters reported in the EAC study.

Specificity

As the expression of chromosomal aberrations is restricted almost exclusively to leukaemic cells and the quality of detection of PCR assays is generally very good, both RT-PCR and RQ-PCR of chromosomal aberrations proved to be highly specific methods for identification of the certain gene rearrangements in leukaemic population in the diagnostic samples as well as detection of MRD in the follow up samples.

Despite that, the use of positive and negative controls with every PCR is still essential; in nested PCR they may help to distinguish true positivity from the occasional occurrence of weak unspecific bands of atypical sizes, in RQ-PCR they may be necessary for correct evaluation of a so called "baseline creeping" artefact [Gabert *et al* 2003].

Sensitivity

Achieved sensitivities of RT-PCR and RQ-PCR were high and corresponding with those reported in BIOMED-1 and EAC studies [van Dongen *et al* 1999, Gabert *et al* 2003]. The sensitivity slightly varies for different chromosomal aberrations and PCR reactions. These variations are mainly caused by the fact that different chromosomal aberrations are expressed in leukaemic cells in different levels of gene transcripts. The structure of a particular fusion gene sequence may allow a design of optimal or less optimal primers and probes that determine the quality of the relevant PCR reaction and may be therefore an additional factor influencing the assay sensitivity.

In positive cell line 10-fold dilution series, the established reproducible sensitivity of first round PCR was $10^{-2} - 10^{-5}$, nested PCR $10^{-4} - 10^{-6}$, and RQ-PCR $10^{-4} - 10^{-6}$.

Overall characteristics of the method

PCR of chromosomal aberrations is probably the best established and standardised method of MRD detection in acute leukaemia and has several significant advantages. The chromosomal aberrations are very specific for leukaemia cells (in healthy individuals they may only rarely be detected in levels $< 10^{-6}$ while a number of LAPs used for FC MRD monitoring may be found in healthy donors in levels around $10^{-3} - 10^{-4}$ and even higher in regenerating bone marrow), in many cases are directly involved in the leukaemogenic process, and are quite stable during the course of the disease. This determines the excellent results of MRD detection by PCR of chromosomal aberrations with the highest specificity and sensitivity among all methods of MRD monitoring. Positive MRD finding also carry the utmost significance for clinical practice, because of the implicated role of chromosomal aberrations in the disease

pathogenesis, some of them being even targets for molecular therapy. Thanks to the international standardisation, the results are very reproducible and well comparable between different centres. The main disadvantage is its limited applicability - chromosomal aberrations could only be detected in less than half of patients with B-ALL and up to one third of patients with T-ALL and AML. It also allows only relative quantification of MRD level, in comparison to the original level at diagnosis. As an RNA-based assay, the levels of the aberrant gene expression may not exactly reflect the amount of the persisting leukaemic cells. The nature of the target, common for all patients with the particular aberration, brings a risk of potential cross-contamination of samples, prevention of which requires a special care.

Future prospectives

Thanks to microarrays and other modern technologies, a number of gene rearrangements have been recently identified in acute leukaemia patients. Some of them, found to be present in a significant proportion of patients, may act as novel suitable MRD markers and potential targets for molecular therapy. The introduction of multiplex PCR reactions for screening of fusion gene transcripts may facilitate the procedures and extend the number of aberrations routinely diagnosed in acute leukaemia patients.

6.2.2. Results of clinical samples analysis

Detection of chromosomal aberrations in diagnostic samples

All B-ALL diagnostic samples (20 patients) were screened for the presence of chromosomal aberrations t(9;22) *BCR-ABL* p190 and p210, t(1;19) *E2A-PBX1*, t(4;11) *MLL-AF4* and t(12;21) *TEL-AML1*. Of these, 8 (40 %) were found positive for t(12;21) *TEL-AML1* translocation; apart from the expression of a standard transcript (in 4 of 8 patients), expression of a variant transcript 39 bp shorter was also detected, either together with the expression of the standard transcript or alone (the remaining 4 of 8 patients). This translocation is the most frequent aberration in childhood B-ALL, although the incidence in large childhood B-ALL population is lower (up to 25 %) [Degos *et al* 2000, Knowles *et al* 2001, van Dongen *et al* 1999, Cazzaniga *et al* 2002a and b, Campana *et al* 2003].

In 1 other patient with B-ALL, the screening RT-PCR for t(4;11) *MLL-AF4* generated a weak band of an atypical size, bigger (between 700 – 800 bp) than sizes of normal products of this reaction. The subsequent confirmatory RT-PCR for this translocation was negative and we therefore considered this patient to be negative for t(4;11) *MLL-AF4* translocation. Later, we compared the results of PCR with other findings obtained for this patient. The results of cytogenetics for this patient did not reveal any chromosomal abnormality, either (Dr. Aengus O'Marcaigh, Department of Haematology, OLHSC Crumlin, personal communication). However, by flow cytometry we found in this patient a strong overexpression of cell-surface chondroitin sulfate proteoglycan molecule known as the rat homologue NG2 antigen, detected by m7.1 monoclonal antibody. This antigen is normally expressed on human glial cells and not present on any cells of haematopoietic origin. Its ectopic expression has been found on subsets of ALL and AML and is correlated to MLL gene rearrangement. The clinical course of the disease in this patient was dismal with early death at the end of induction treatment (Dr. Aengus O'Marcaigh,

Department of Haematology, OLHSC Crumlin, personal communication); such a poor prognosis is typically associated with *MLL* gene alterations. We therefore conclude that this patient may have had an *MLL* gene rearrangement other than t(4;11) *MLL-AF4*.

No chromosomal aberrations t(9;22) *BCR-ABL* p190 and p210 or t(1;19) *E2A-PBX1* were found in B-ALL diagnostic samples. Translocations t(9;22) *BCR-ABL* p190 and p210 together with t(4;11) *MLL-AF4* are infrequent in B-ALL (each present in about 5 % of cases) [Degos *et al* 2000, Knowles *et al* 2001, van Dongen *et al* 1999, Cazzaniga *et al* 2002b, Campana *et al* 2003] so their absence in the limited number of examined samples was as expected. The translocation t(1;19) *E2A-PBX1* has an incidence in childhood B-ALL about 5 – 15 % [Degos *et al* 2000, Knowles *et al* 2001, van Dongen *et al* 1999, Cazzaniga *et al* 2002b, Campana *et al* 2003]. The non-representative character of our small group of B-ALL patients can explain the slight differences in occurrence of particular aberrations among our patients in comparison with their frequencies in a large childhood B-ALL population – like a lower occurrence of t(1;19) *E2A-PBX1* translocation, and on the contrary a higher occurrence of t(12;21) *TEL-AML1* translocation.

The T-ALL samples (3 patients) were screened for microdel (1p) *SIL-TAL1*, and AML samples (2 patients) for t(8;21) *AML1-ETO*, t(15;17) *PML-RARA* (bcr 1, 2 and 3), and inv(16) *CBFB-MYH11* (all the main types), but no chromosomal aberration was detected in these samples. This reflects their relatively low occurrence in T-ALL or AML, respectively - microdel (1p) *SIL-TAL1* is found in about 20 % of T-ALL; all the aberrations in AML together constitute about 30 % of AML cases [Degos *et al* 2000, Knowles *et al* 2001, van Dongen *et al* 1999, Cazzaniga *et al* 2002b, Campana *et al* 2003, Liu Yin *et al* 2002, Grimwade *et al* 2002, Lo-Coco *et al* 2003a and b].

Monitoring of MRD in follow-up samples

MRD was detected by RQ-PCR quantification of *TEL-AML1* transcripts in 7 B-ALL patients (in 1 patient no follow up samples were available). Only 2 of them (29 %) achieved MRD negativity by day 29; 4 were positive with low or very low MRD levels, 1 patient had no D29 sample available. Most of these patients (4; 57 %) showed MRD clearance by week 7 – 12, only 1 patient (14 %) remained very weekly positive and reached MRD negative status in the next samples. All the patients remained MRD negative during the study follow-up. This pattern of MRD clearance is comparable with findings of other studies monitoring MRD by PCR of fusion gene transcripts [Cimino *et al* 2000, Gamiero *et al* 2002, Neale *et al* 2004].

6.3. Detection of minimal residual disease in childhood acute leukaemia by PCR of clonal IG/TCR rearrangements

6.3.1. Methodology

The methods of detection of clonal *IG/TCR* rearrangements and their application in MRD monitoring were set up according to the recommendations of BIOMED-2 group and the reference studies [Pongers-Willemse *et al* 1998, van Dongen *et al* 2003, Verhagen *et al* 2000, Langerak *et al* 2001, van der Velden *et*

al 2002b and c, Bruggemann *et al* 2004]. All steps were optimised and validated on control samples and the parameters of the assays checked to verify their efficiency, specificity, sensitivity and reproducibility. The results were compared with results of other studies employing PCR of clonal *IG/TCR* rearrangements in MRD detection [Cave *et al* 1998, van Dongen *et al* 1998, Panzer-Grumayer *et al* 2000, Biondi *et al* 2000, Willemse *et al* 2002, Nyvold *et al* 2002, Scrideli *et al* 2002, Marshall *et al* 2003].

PCR screening of clonal IG/TCR rearrangements

The PCR screening for *IG/TCR* clonality using multiplex assays from the BIOMED-2 protocols [van Dongen *et al* 2003] is simple and efficient. There are 3 options for detection of the clonal products: heteroduplex analysis, electrophoresis on high-resolution agarose and by capillary electrophoresis (GeneScan). Heteroduplex analysis has been recognised as a "gold standard" in detection of clonality products. After denaturation (at high temperature) and renaturation (at lower temperature), PCR products of clonally rearranged genes give rise to homoduplexes, whereas in case of polyclonal products heteroduplexes are formed. They are separated based on their conformation in non-denaturing polyacrylamide gels [Langerak *et al* 1997]. This method was used in our department at the initial phases of the whole MRD project. Later, the other newer methods were tested and showed results comparable to the heteroduplex analysis (Fiona Quinn, Cancer Molecular Diagnostics, St. James's Hospital, personal communication). Because of the biohazard of the work with polyacrylamide (high toxicity), we then used the other two methods for clonality products detection and heteroduplex analysis would be employed only in case when these techniques could not reach a conclusion. Electrophoresis on high resolution agarose gel is probably the easiest way of clonal products analysis and under optimised conditions (lower voltage, longer time, lower temperature than for standard gel electrophoresis) can give quite accurate results. However, in few cases the distinction between mono- and polyclonal products may not be quite clear. Capillary electrophoresis (GeneScan) requires certain equipment. It can generate very precise results and make the decision when the results of gel electrophoresis are ambiguous. The main disadvantage is that it cannot separate the products when more than one monoclonal peaks are present. In such cases, the PCR product has to be run on the high resolution agarose and the monoclonal bands cut out of the gel.

Sequencing of the clonal products and their analysis

The monoclonal products were sequenced in a standard way. The resulting sequences were compared with databases – a number of them are available at present, the most widely used is probably that of the National Library of Medicine of the National Institute of Health (BLAST, IGBLAST).

ASO primers design and testing

The main advantage of ASO-primer over ASO-probe approach [Pongers-Willemse *et al* 1998] is its feasibility, lower costs and theoretical higher sensitivity (no competition contrary to the use of germ-line primers), and is therefore used in many studies on MRD detection by *IG/TCR* rearrangements [Verhagen *et al* 2000, Langerak *et al* 2001, Malec *et al* 2001 and 2004, van der Velden *et al* 2002b and c, Marshall *et al* 2003, Neale *et al* 2004, Bruggemann *et al* 2004, Cazzaniga *et al* 2005]. According to the common recommended criteria, 2 – 4 ASO primers per one patient were designed and tested. While for all patients

with *IGH* or *TCRD* rearrangements it was easy to find a well-working specific primer, we were successful only in over half of the patients (5 of 9) with *IGK-Kde* rearrangements. This is probably caused by the fact that only one junctional region is present in this immunoglobulin chain and it seems to be often involving long CG sequences or, on the other hand, very few (1 - 2) N-nucleotides, which both precludes finding a primer that would work with high enough sensitivity and specificity.

Detection of MRD by RQ-PCR

The RQ-PCR detection and quantification of clonal rearrangements was performed as described previously [Verhagen *et al* 2000, Langerak *et al* 2001, van der Velden *et al* 2002b and c, Bruggemann *et al* 2004], the assay performed well, no special optimisation was necessary .

Applicability

With this method, MRD could be detected in the vast majority of ALL patients – 12 out of 13 patients with B-ALL (92 %) and the only one patient with T-ALL. In half of the B-ALL patients, 2 independent *IG/TCR* targets were found and followed. In B-ALL, most of the clonal targets used for MRD monitoring were *IGH* complete and incomplete rearrangements (72 %), *IGK-Kde* were applicable only in about a quarter of the cases (28 %). As said previously, these rearrangements were present more frequently (in 9 of 13 patients) but the specific RQ-PCR reaction could be set up only in some of them. The T-ALL patient was monitored by *TCRD* clonal rearrangement. These results, though obtained from a quite small group of ALL patients, support well the fact that MRD can be monitored by detection of clonal rearrangements in the majority of ALL patients (over 90 % of both, B- and T-ALL) [Pongers-Willemse *et al* 1999, Verhagen *et al* 2000, Langerak *et al* 2001, van der Velden *et al* 2002a, b, c, 2003 and 2004b, Szczepanski *et al* 2000, 2001b, 2002a and b, Li *et al* 2001, Cazzaniga *et al* 2002a and 2005, van Dongen *et al* 2003].

Specificity

The clonal rearrangements in *IG* or *TCR* genes are quite unique and patient-specific. However, similar to the expression of LAPs detected by FC, they may not be quite stable during the disease course and may be prone to subclone formation, or clonal evolution with secondary rearrangements and mutations. Rearrangements of *TCRs* are generally more stable (particularly *TCRG* in T-ALL) than rearrangements of *IGs*; D - J rearrangements are more stable than V- DJ joint regions; monoclonal rearrangements are more stable than oligoclonal. *IGK-Kde* rearrangements, as they represent the end-stage change, are recognised as the most stable MRD targets [Szczepanski *et al* 2002b and 2003, van der Velden *et al* 2004b, Germano *et al* 2003, Cazzaniga *et al* 2005]. Because the loss of the original rearrangement may cause false negative results, 2 – 3 independent clonal targets should be preferentially used for a reliable MRD monitoring. We used 2 independent *IG/TCR* targets in all cases where no other MRD markers were available. In patients monitored simultaneously by PCR of fusion genes and/or FC of LAPs, only one clonal target was sometimes used.

Otherwise, the specificity of the reaction is determined by the specificity of the designed ASO primer. This depends, most of all, on the length and sequence of the N-junctional region. Very short junctional

regions or those containing long CG repeats may unable a design of primers with sufficient specificity and sensitivity.

The specificity of the RQ-PCR is tested against the polyclonal background and only ASO primers with no or very weak positivity with polyclonal DNA (coming up around 40^{th} cycle) are approved for the MRD detection.

Sensitivity

The overall sensitivity of the RQ-PCR was $\geq 10^{-4}$, which is in agreement with the recommendations and requests of the relevant studies [Pongers-Willemse *et al* 1998, van Dongen *et al* 2003, Verhagen *et al* 2000, Langerak *et al* 2001, van der Velden *et al* 2002b and c, Bruggemann *et al* 2004]. The sensitivity is, again, defined by the performance of the ASO primers and the difference against the polyclonal background. The achieved sensitivities of the individual reactions were between $10^{-4} - 10^{-5}$ (of the 10-fold dilution series of the patient's diagnostic sample) for all patients.

Overall characteristics of the method

PCR of clonal *IG/TCR* rearrangements is the most widely used method of MRD detection in ALL. Its main advantage is the broad applicability enabling MRD detection in over 90 % of ALL patients. The use of 2 or more independent targets ensures a reliable specificity of the results, with a very good sensitivity $10^{-4} - 10^{-5}$. It's a well standardized method, and although the quantification is relative (in terms of expression of MRD as a percentage of the level in the diagnostic sample), as a DNA-based assay it reflects quite precisely the amount of the residual leukaemic cells. The targets are patient-specific, which prevents a risk of potential cross-contamination. The main drawbacks are the demands associated with this method on the staff and equipment. It is a rather laborious multi-step method that takes a certain time to perform, requires trained staff and use of different laboratory techniques, which increases the costs of the method.

Future prospectives

Novel techniques and approaches could simplify the detection of *IG/TCR* clonal rearrangements and make its use easier. These include the PCR clonality screening with multiplex reactions, detection of the clonal products by capillary electrophoresis (GeneScan), or use of specific software products for the ASO primer design. Also recently, monoclonal rearrangements *TCRD/A* (Vd2-Ja) were described as novel reliable clonal targets in B-ALL patients because of their high sensitivity and good stability [Cazzaniga *et al* 2005].

6.3.2. Results of clinical samples analysis

Detection of clonal IG/TCR rearrangements in diagnostic samples

Of the 14 ALL patients (13 B-ALL, 1 T-ALL) with available diagnostic DNA samples, at least one clonal *IG/TCR* target suitable for MRD monitoring was identified in 13 (93 %) of them (12 B-ALL, 1 T-

ALL). Half of the B-ALL patients were monitored by 2 independent clonal markers, the remaining B-ALL and the one T-ALL patients were followed by 1 clonal marker – in all these patients MRD was detected by at least one other method.

In B-ALL, the most widely used clonal markers were *IGH* complete and incomplete rearrangements (61 and 11% of all rearrangements, 72 % in total); *IGK-Kde* rearrangements comprised less than one third (28 %). *IGH* complete and incomplete rearrangements were thus detected in 78 and 8 % of B-ALL childhood patients (86 % in total), *IGK-Kde* in 38 % of them. The T-ALL patient expressed *TCRD* clonal rearrangement. These findings are comparable with previously published data [Pongers-Willemse *et al* 1999, Verhagen *et al* 2000, van der Velden *et al* 2002c, Cazzaniga *et al* 2002a, Szczepanski *et al* 2002a and b, van Dongen *et al* 2003], slight differencies could be explained by our small group of patients investigated. *IGH* rearrangements are described as the most frequent *IG/TCR* targets in childhood B-ALL (about 95 % of cases, 80 % complete and 15 % incomplete). They are usually quite easy to identify and also the ASO primer design is usually successful. However, they are at the same time one of the least stable *IG/TCR* targets. *IGK* rearrangements are less frequent, being found in up to 60 % of childhood B-ALL, and their detection may be a bit cumbersome because of difficulties in distinguishing monoclonal and polyclonal rearrangements in a proportion of cases. On the other hand, *IGK-Kde* rearrangements are considered as very valuable because of their high stability during the disease course. In childhood T-ALL, the most common rearrangements are *TCRG* (90 %), also very stable, followed by *TCRD* rearrangements (about 60 %).

Monitoring of MRD in follow-up samples

MRD was monitored in 12 of 13 patients with the specific RQ-PCR established. Over half of these patients (7; 58 %) reached MRD negativity by day 29. Two further patients (17 %) with low and very low MRD levels on day 29 showed MRD clearance by week 7 – 12. In 3 patients (25 %), MRD negativity was achieved only later, after this time-point. Two of them had significant levels of MRD in day 29 and week 7 – 12 samples. In one of the patient with very low MRD level on day 29 and MRD negativity on week 7 – 12, an isolated very weak MRD positivity (around the detection sensitivity threshold) was found in one of the later samples (week 34), all the previous and consequent samples were negative. No reoccurrence of MRD was noticed in the remaining patients once they achieved MRD negativity. Of the 13 patients, one patient died of the disease before day 29 and his MRD levels in day 8 and 15 samples were very high. This patient with T-ALL showing an uncommon immunophenotype was stratified at diagnosis to group B (because of peripheral leukocytosis) but had an atypical course of the disease with absolutely no response to the initial therapy (Dr. Aengus O'Marcaigh, Department of Haematology, OLHSC Crumlin, personal communication). The treatment response and course of MRD levels in the other patients were as expected and in agreement with results of other authors [Cave *et al* 1998, van Dongen *et al* 1998, Panzer-Grumayer *et al* 2000, Biondi *et al* 2000, Willemse *et al* 2002, Nyvold *et al* 2002, Scrideli *et al* 2002, Marshall *et al* 2003].

Correlation of MRD presence and levels detected in the same patients by different IG/TCR clonal targets

In the patients where 2 different *IG/TCR* clonal markers were used for MRD detection, the correlation of their results was found to be excellent (Spearman rank test - $p < 0.0001$). The only discrepancies present were of minor character and in the lowest MRD levels, close to the detection threshold. However, as there was no relapse detected in the monitored patients during the study follow-up, no conclusion in terms of stability and reliability of these markers at relapse could be made.

6.4. Correlation of MRD results between different methods of MRD detection and with clinical data

6.4.1. Applicability and correlation of different methods of MRD detection

Applicability of different methods of MRD detection

MRD could be monitored by at least one method in 18 of 25 patients enrolled in the study. Four of the 16 ALL patients (25 %) were followed by one method only – half of them by FC, the other half by PCR of clonal *IG/TCR* rearrangements. Combined monitoring by FC and PCR (of chromosomal aberration or clonal *IG/TCR* rearrangements) was applicable in 7 patients (44 %) and all three approaches of MRD detection (FC and PCR of chromosomal aberration and PCR of clonal *IG/TCR* rearrangements) could be used in 5 patients (31 %). The 2 AML patients did not express any of the screened chromosomal aberrations and both could be monitored by FC only.

The vast majority of patients could be followed by flow cytometry - suitable LAPs as MRD targets were found in all patients in whom diagnostic or day 8 samples for flow cytometry were available. These represented in total 16 out of 23 (70 %) patients with ALL and both 2 AML patients enrolled to the study. PCR of clonal *IG/TCR* rearrangements could be applied in most of ALL patients - 13 out of 14 patients (93 %) in whom the diagnostic DNA was available, and of 23 ALL patients in total (57 %). Only 8 of 25 (32 %) patients, all of whom were screened, had a chromosomal aberration that could be used for MRD monitoring by PCR of fusion gene transcripts.

It seems that the crucial factor influencing the applicability of the different methods of MRD detection is the quality of the sample upon its arrival to the lab. In the 7 of 25 patients who could not be monitored for MRD status, the main problem was that either no diagnostic sample was available (and no MRD markers could be found in day 8 sample), or no follow-up samples were obtained. Although the bone marrow biopsy in a small proportion of childhood acute leukaemia patients is difficult with very low yields of cells, the main reason why samples are not sent to the lab are rather organizational problems.

If no diagnostic sample is available, the search for suitable MRD markers could be performed in day 8 sample, which we have done several times. In this case, the easiest and most specific technique is the PCR screening for chromosomal aberrations, as this method is very specific and sensitive and thus able to identify the marker (once it is present) even in a sample with a considerable reduction of leukaemic cells by the initial therapy. Because of the universal character of some LAPs (like CD58 or CD34/38/45 expression), the day 8 sample can be also successfully screened by FC. But detection of MRD markers by

FC will be complicated or even impossible if the cellularity of the sample or the proportion of the leukaemic cells in the sample are very low. Similarly, MRD markers identification by PCR of clonal *IG/TCR* rearrangements in day 8 sample gives reliable results only if the fraction of the leukaemic cells in the sample is still significant. Otherwise the assessment of monoclonal products on strong polyclonal background may be difficult and, in case of biallele or biclonal rearrangements, some of them could be missed. We managed to identify MRD markers in all day 8 samples available for flow cytometry and PCR of chromosomal aberrations but no day 8 samples available for PCR assessment of clonality. This explains why more ALL patients in our study could be followed by FC than by PCR of clonal rearrangements.

In some cases, samples arriving to the lab are of rather poor quality with low amount of viable bone marrow cells. The hypocellularity of the samples may be due to the bone marrow aplasia occurring during the course of anti-leukaemic therapy, especially in particular time-points. However, in some cases it could be also caused by technical problems during bone marrow aspiration and transport of the sample, like contamination of a bone marrow sample with peripheral blood during the aspiration, partial coagulation of the sample or prolonged transfer of the sample to the lab.

Correlation of different methods of MRD detection

The correlation of MRD presence and levels detected by different targets within and in between the several methods showed a very good overall correlation but certain differencies could be noticed. While the use of different *IG/TCR* clonal rearrangements in the same patients brought almost identical MRD results, the use of different LAPs detected by flow cytometry in the same patients showed more pronounced discrepancies. This probably reflects the characteristics of the particular methods with, in certain cases, more subjective definition and assessment of LAPs and slightly lower sensitivity of flow cytometry in comparison with PCR of clonal rearrangements.

Comparison of MRD results generated by the two PCR methods – detection of clonal *IG/TCR* rearrangements and fusion gene transcripts – gave very precise correlation not only in terms of the presence but also of the quantity of MRD levels. The correlation between flow cytometry and PCR methods showed also a very good level of agreement, though not as perfect as in the previous case. Overall, the correlation within and in between the three methods of MRD detection was always highly significant with $p < 0.0001$ in Spearman rank test; the individual discordancies were rare and confined to low levels of MRD (mostly below 10^{-3} or 0.1 %). Our results support findings of previously published studies [Chen *et al* 2001a, Gameiro *et al* 2002, Malec *et al* 2001 and 2004, Coustan-Smith *et al* 2003, Neale *et al* 2004, Kerst *et al* 2005, Dworzak *et al* 2003, van der Velden *et al* 2004b] indicating a good correlation between different methods of MRD detection but admitting the existence of certain discrepancies, which so far precludes a direct comparison or matching of the MRD data generated by different techniques.

6.4.2. Correlation of MRD results with clinical data

When looking for the clinical relevance of MRD monitoring, the main objectives of interest are the risk of relapse and relapse-free survival in relation to the presence and level of MRD. In this study, no case of relapse appeared, probably due to the limited number of patients and length of follow-up, so no conclusion on the predictive value of the measured MRD levels in our patients could be drawn. Of 16 ALL patients, 7 (44 %) reached MRD negativity by day 29, and all of them had MRD levels on day 15 below 0.1 % of the initial values in their diagnostic samples. In these patients, the prognosis should be favourable and the risk of relapse low. Interestingly, they belonged to all risk groups (according to the risk stratification at diagnosis), and 2 of them died of sepsis in the first six months after diagnosis. We might speculate if, in the future, MRD-adjusted therapy with reduced intensity could achieve persisting remission in these patients and prevent occurrence of the fatal complications of anti-leukaemic treatment in some of them. On the other hand, 3 patients (19 %) achieved MRD negativity only after week 12; 2 of them had also very high MRD levels in day 15 and day 29 samples (over 10 % and 0.1 % of the level at diagnosis, respectively). In addition, one patient who died of leukaemia progression before the first main follow-up time-point (day 29) had very high MRD levels in day 8 and day 15 samples. Also these patients were stratified into various risk groups at diagnosis but according to the MRD levels their prognosis is supposed to be unfavourable and their risk of relapse may be quite high. In the remaining 5 patients (31 %), who became MRD negative between day 29 and week 12, the predictive value of the MRD status could be considered as unclear.

Such MRD risk prediction is suggested by many of the published studies. The clinical relevance of MRD was proven in newly diagnosed acute leukaemia as well as relapsed acute leukaemia and transplanted patients (see chapter 2.3.3.2. and table 2.24 for overview of the studies, reviewed in Szczepanski *et al* 2001b, Hoelzer *et al* 2002, Cazzaniga *et al* 2002a and 2005, Campana *et al* 2003, Dworzak *et al* 2003, van der Velden *et al* 2003 and 2004b). Though all the large-scale studies demonstrated the independent prognostic significance of MRD in childhood ALL and AML, there are differences in the MRD levels, their cut-off values and time-points used for the risk prediction and patients stratification across the studies. However, in summary we could say that in childhood ALL MRD negativity at the end of induction (day 29 – 35 in different treatment protocols) or even earlier, in the middle of induction on day 15, define MRD low risk group (about 40 % of patients) with a good prognosis and low risk of relapse. These patients could potentially benefit from therapy reduction. On the other hand, MRD > 0.1 % at the end of induction and > 0.01 % before consolidation (week 7 - 12) represent MRD high risk group (about 10 % of patients) with unfavourable prognosis, a high relapse rate and frequent early relapses, and these patients may benefit from intensified treatment. The remaining patients (50 %) belong to the MRD intermediate risk group with occasional and often late relapses and the treatment approach to them will have to be further considered.

Finally, it has to be emphasized that the potential clinical benefit of MRD monitoring in childhood acute leukaemia patients requires confirmation in prospective large-scale clinical trials, which are currently being performed and results will be available shortly. Their results will be probably influenced by applicability and reproducibility of the methods of MRD detection (required high specificity and

sensitivity may need to be weighed with the respect to the diagnostic aim – to diagnose the patients with a good response and stratify them to MRD-low risk group with possible treatment reduction, the highest sensitivity of the methods is important and false-positive MRD results are less significant than false-negative; for diagnosis of patients with resistant leukaemic disease belonging to MRD-high risk group and requiring intensified treatment, the sensitivity around 10^{-4} or 0.01 – 0.05 % may be sufficient while the request on very high specificity is the priority), the progress in the field of anti-leukaemic therapy (how much can the intensified therapy improve the relapse rate in patients from MRD high-risk group, how much can the reduced therapy decrease the incidence of treatment complications while still keeping the risk of relapse low in the patients from the MRD low-risk group, all together how much can the MRD-tailored treatment improve the overall survival and quality of life of the patients), and of importance will also be the cost-effectiveness aspect of this approach.

6.5. Detection of WT1 expression in childhood acute leukaemia by RT-PCR and RQ-PCR

6.5.1. Methodology

RT-PCR for total WT1and its isoforms

The RT-PCR detection of total *WT1* and its four main isoforms we designed, established and optimised according to the BIOMED-1 protocols for detection of chromosomal aberrations in acute leukaemia [van Dongen *et al* 1999, Gabert *et al* 2003], with knowledge of the published studies on RT-PCR detection of *WT1* expression in acute leukaemia [Menssen *et al* 1995, Inoue *et al* 1997, Gaiger *et al* 1999, Cilloni *et al* 2002, Garg *et al* 2003, Niegemann *et al* 1999, Ozgen *et al* 2000, Ogawa *et al* 2003]. All the reactions were validated on groups of control and patients' samples. The parameters of the reactions, their specificity, sensitivity and reproducibility, fulfil the BIOMED-1 criteria for RT-PCR analysis of chromosomal aberrations and MDR detection in leukaemia.

The established RT-PCR for *WT1* and its isoforms could be therefore proposed as standard qualitative PCR assays for detection of *WT1* in acute leukaemia. The design of the reactions also allows simultaneous detection of *WT1* expression and chromosomal aberrations in leukaemia patients.

RQ-PCR for total WT1 and its isoforms

The RQ-PCR for detection and quantification of total *WT1* and *WT1* isoforms expression was designed, established and optimised according to the EAC protocols for analysis of fusion gene transcripts and MRD in leukaemia [Gabert *et al* 2003, Beillard *et al* 2003], with knowledge of the published studies on RQ-PCR detection of *WT1* expression in acute leukaemia [Kreuzer *et al* 2001, Cilloni *et al* 2002 and 2004, Trka *et al* 2002, Tamaki *et al* 2003, Ogawa *et al* 2003, Uzunel *et al* 2004, Ostergaard *et al* 2004, Dumur *et al* 2002]. As in case of RT-PCR, the reaction was validated on groups of control and patients' samples. The parameters of the reaction, its efficiency, specificity, sensitivity and reproducibility, fully comply with the EAC criteria for detection of fusion gene transcripts and MRD. However, because of the

discrepancy between the detected KTS-positive isoforms and total *WT1* found in many of the studied patients, the reaction for detection of KTS isoforms will be further evaluated.

The established RQ-PCR for total *WT1* and its exon 5-positive isoforms could be considered as a standard reaction for their detection and quantification in acute leukaemia. Multicenter testing with quality control study and assessment of the stability of *WT1* expression in different samples may be useful to complete our work on the standardisation of RQ-PCR for *WT1* and its isoforms. Although the method has been set up for TaqMan technology, it may be applied to other types of RQ-PCR instruments.

The results are expressed as normalized WT1 copy number and have been calculated from the standard curve method. The comparison of WT1 and CG standard curves by validation analysis confirmed their equal efficiency, which allows using the ΔΔCt method for *WT1* quantification with its possible advantages (reduction of costs, reduction of a risk of contamination, testing more samples on a single plate). When the *WT1* expression levels are assessed in a sufficient number of normal samples, one reference containing WT1 and CG copy numbers that represent the mean level of *WT1* and CG expression in normal bone marrow could be prepared for this purpose. In the future, such a reference may also represent a standard that would enable quick evaluation of *WT1* overexpression without the necessity of previous testing large amounts of control normal samples in each individual research centre. The sensitivity levels of the individual experiments may be calculated according to the EAC recommendations [Gabert *et al* 2003].

Specificity

All the RT- and RQ-PCR reactions for *WT1* have been designed and confirmed in validation tests to detect specifically total *WT1* or its isoforms. As a variable level of *WT1* expression can be found in all haematopoietic tissues, *WT1* expression, contrary to chromosomal aberrations, does not represent a specific marker of leukaemic cells. High levels of *WT1* expression in bone marrow or peripheral blood samples are, however, associated with the presence of leukaemic population [Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002, Kreutzer *et al* 2001, Cilloni *et al* 2002], despite the fact that *WT1* expression in single haematopoietic stem cells can reach a degree similar to that of single leukaemic cells [Maurer *et al* 1997, Ellisen *et al* 2001, Hosen *et al* 2002].

Sensitivity

Sensitivity of all PCR reactions for *WT1* – RT-PCR and RQ-PCR for *WT1* and its isoforms – was high and comparable with that reported in BIOMED-1 and EAC studies for RT- and RQ-PCR of chromosomal aberrations and fusion gene transcripts [van Dongen *et al* 1999, Gabert *et al* 2003].

With K562 cell line 10-fold dilution series, the reproducible sensitivity reached 10^{-3} for first round and 10^{-5} for nested RT-PCR of total *WT1*, 10^{-2} – 10^{-3} for first round and 10^{-4} for nested RT-PCR of *WT1* isoforms, and 10^{-5} for RQ-PCR of *WT1* and its isoforms. With a series of plasmid calibrators, the reproducible sensitivity of RQ-PCR of *WT1* and its isoforms was 10^{1} gene copies. For patients' samples, the sensitivity varied according to their level of *WT1* expression. In those with *WT1* overexpression, the achieved sensitivity was 10^{-3} for first round RT-PCR of total *WT1*, and 10^{-4} for the nested reaction and RQ-PCR.

Correlation of RT-PCR and RQ-PCR for WT1

The RT-PCR and RQ-PCR methods showed very good overall correlation in detection of *WT1* expression. The reproducible sensitivity of nested PCR and RQ-PCR was comparable, as tested on K562 dilution series, 10^{-5} for each of the methods) as well as on clinical samples (*WT1* detected in all samples tested by each of the methods). None of the methods has showed any unspecific positivity during the testing. By qualitative assessment, the intensity of the bands in PCR reactions corresponded to the levels of *WT1* expression detected by RQ-PCR.

Overall characteristics of the method

PCR of *WT1* is a novel promising method detecting a potential prognostic factor and MRD marker in acute leukaemia. It is possible to standardise this assay in the same way as PCR of chromosomal aberrations and thus obtain very reproducible and consistent results between experiments as well as between different centres. Because the discrimination of normal haematopoietic cells from leukaemic cells according to *WT1* expression has a quantitative character, initial assessment of *WT1* expression in a large group of control samples is necessary to define the ranges and average of *WT1* expression in normal haematopoietic tissues. However, if a standardised assay for *WT1* detection is used, the values of *WT1* expression in normal haematopoietic cells, once established, may be used as a reference for other centres, excluding the demands on testing a control population by each individual laboratory. The significance of *WT1* as a prognostic factor and MRD marker in acute leukaemia is at present being tested in several ongoing clinical studies. The preliminary results suggest that *WT1* may be an independent prognostic factor in both ALL and AML [Sugiyama *et al* 1998, Menssen *et al* 2002, Trka *et al* 2002, Ogawa *et al* 2003, Ramirez *et al* 003, Cilloni *et al* 2002]. At the moment, it also appears that *WT1* may be the most convenient MRD marker in AML, where *WT1* overexpression could be used for MRD monitoring in about 50 % of cases [Trka *et al* 2002, Cilloni *et al* 2004, Ostergaard *et al* 2004]. This is of particular importance as the applicability of PCR of chromosomal aberrations in detection of MRD in AML is limited up to 30 % of patients, and FC detection of MRD in AML remains problematic, being the least standardised method with limited specificity and sensitivity, requiring representative group of controls and certain experience in analysis of the results. Similar to PCR of chromosomal aberrations, PCR of *WT1* also offers only relative quantification of MRD level, and carries a risk of potential cross-contamination of samples that has to be prevented by a good laboratory practice.

Implications of the standardised RT-PCR and RQ-PCR assays for further research on WT1 expression in acute leukaemia

As the RT-PCR and RQ-PC assays for *WT1* detection we have established fulfil all the required criteria, we would recommend them as standard methods for detection of *WT1* expression.

Standardised PCR detection of *WT1* is a necessary prerequisite for further studies on *WT1* expression and its use for MRD monitoring in acute leukaemia where it could help to address issues that still remain partially unresolved or controversial. These include (1) the mean values and the maximal ranges of *WT1*

expression in normal and control haematopoietic tissues - normal bone marrow, normal peripheral blood, peripheral stem cell concentrate, umbilical cord blood, MRD negative bone marrow at various stages of regeneration after chemotherapy and pre- and post-transplant bone marrow; (2) the mean values and the maximal observed ranges of *WT1* expression in diagnostic bone marrow and peripheral blood of different types and subtypes of acute leukaemia, and in bone marrow with other malignant and non-malignant haematological diseases; (3) the significance of *WT1* as an independent prognostic factor in different types and subtypes of acute leukaemia, and its potential for differential diagnosis of certain haematological disorders; (4) the suitability of *WT1* for MRD monitoring in different types and subtypes of acute leukaemia, particularly in terms of its applicability, specificity and sensitivity; (5) the suitability of detection of *WT1* in peripheral blood for MRD monitoring or differential diagnosis; (6) the differences in *WT1* expression in childhood and adult, both in normal donors and in acute leukaemia patients; (7) the expression pattern of *WT1* isoforms in normal haematopoietic tissues, acute leukaemia and other haematological conditions, its value for prognosis, differential diagnosis and MRD monitoring in acute leukaemia; and (8) the stability of *WT1* expression over time, during sample processing, and in relation to the control genes.

6.5.2. Results of clinical samples analysis

Detection of WT1 and its isoforms in control samples from normal haematopoietic tissues

To evaluate the normal background *WT1* expression, we analysed *WT1* in control samples – PB, BM and PSC from healthy adult donors and MRD-negative regenerating BM from childhood ALL patients. By qualitative RT-PCR assays, *WT1* expression could be detected in all normal controls – bone marrow and peripheral blood. In normal bone marrow, the expression of total *WT1* could be in most cases detected already by first round PCR, nested PCR then confirmed it in all control BM samples. In normal peripheral blood, the total *WT1* expression could be detected in all samples but only by nested PCR, first round PCR was always negative. RQ-PCR of *WT1* correlated fully with the findings of RT-PCR, and detected *WT1* expression in all control samples tested. The median *WT1* expression in PB was approximately 1 log lower than in BM, in PSC *WT1* expression corresponded to values found in normal PB. *WT1* expression in regenerating BM of childhood ALL patients was very similar to that of normal bone marrow of adult donors. Though in MRD-negative BM taken during the induction therapy of childhood ALL *WT1* tended to reach lower levels, *WT1* expression in BM taken during the later phases of anti-leukaemic therapy was practically identical to normal BM and no significant difference in *WT1* expression between the normal and regenerating BM could be observed.

WT1 is physiologically expressed in normal haematopoietic cells, with a typical expression pattern and well-defined function [Ellisen *et al* 2001, Hosen *et al* 2002, Loeb *et al* 2003]. Except for very early investigations [Menssen *et al* 1995], *WT1* expression has been found in normal BM and PSC controls or CD34+ concentrates in all further studies, either in a proportion [Inoue *et al* 1997, Kletzel *et al* 2002, Garg *et al* 2003, Barragan *et al* 2004] or all [Cilloni *et al* 2002, Ostergaard *et al* 2004, Ogawa *et al* 2003] of the samples tested. *WT1* expression in normal PB has not been specifically examined and the function of *WT1* in PB cells is not known. While some authors did not find any *WT1* expression in normal PB

[Menssen *et al* 1995, Kreuzer *et al* 2001, Trka *et al* 2002, Magyarosy *et al* 2003, Barragan *et al* 2004], others detected the presence of *WT1* in a proportion of samples [Cilloni *et al* 2002, Kletzel *et al* 2002, Ostergaard *et al* 2004, Uzunel *et al* 2003]. *WT1* expression in regenerating BM has been assessed mostly indirectly, by evaluation of *WT1* levels in follow-up samples for the purpose of MRD monitoring [Trka *et al* 2002, Garg *et al* 2003, Ostergaard *et al* 2004]. From these studies it seemed that there is no major difference in *WT1* expression in normal BM and BM during various stages of anti-leukaemic treatment. In addition, Cilloni *et al* [Cilloni *et al* 2002] found very similar *WT1* expression pattern in examined normal and regenerating BM samples.

If we analyse all the above papers we could conclude that the proportion of control normal haematopoietic samples tested positive for *WT1* expression seems to be directly related to the quality of the method of *WT1* detection employed. With the increasing specificity and sensitivity of the methods in the recent studies, *WT1* expression could be confirmed in all normal, regenerating and post-transplant BM and in many normal PB samples. Our own results correspond quite well with those reported by Cilloni *et al* [Cilloni *et al* 2002], who employed an RQ-PCR technique for *WT1* detection similar to ours. The levels of *WT1* expression (22 – 115 WT1/ABL x 10^{-4}) we could detect in normal BM are entirely within the ranges detected in this study (3 – 180). The median *WT1* expression in normal BM in our samples was slightly lower than in Cilloni's study (29 vs. 78), which could be explained by our smaller group of examined samples, and possibly also by subtle differences in the methodology. The *WT1* values in normal BM are higher than those referred by several other authors who also used the WT1/CG NCN approach for *WT1* quantification [Garg *et al* 2003, Barragan *et al* 2004, Ostergaard *et al* 2004]. We also found *WT1* expression in normal PB very similar to that reported by Cilloni (0.2 – 13, with median 2 vs. 1 – 22, median 4) and higher than other authors observed [Ostergaard *et al* 2004, Uzunel *et al* 2003]. However, we were able to detect *WT1* in all PB samples unlike Cilloni (only 19 of 33 PB samples were positive for *WT1* in their study) and the other authors. This could be again partially explained by the small number of samples we tested but may also reflect a higher sensitivity of our method of *WT1* detection.

Presence of all main *WT1* isoforms was detected by RT-PCR and RQ-PCR in normal BM, PSC and PB. First round PCR of BM sample gave negative results for variants A and C and was marginally positive for variants B and D, corresponding with the slightly higher sensitivity of PCR assays for these variants. With nested PCR, all four variants were found in normal BM. Normal PB was negative for all *WT1* variants on first round PCR; with nested PCR, only variants C and D, containing KTS fragment, were detected in the PB sample (but only 1 sample was tested by this method so far). By RQ-PCR, exon 5-positive and KTS-positive isoforms were found in all control samples. While exon 5-positive isoforms were detected in levels slightly lower than total *WT1*, KTS-positive isoforms were found in normal BM slightly and in normal PB significantly higher than total *WT1*. When looking for possible explanations of this unexpected discrepancy, a technical problem or error in *WT1-KTS* detection was considered at the first stage. Any unspecifity or cross-reactivity of the reaction were excluded. However, due to the character of the sequence around KTS region, the reaction was designed in such a way that both primers and probe were localised within one exon, exon 9 (including its KTS fragment), while the reaction for total *WT1* and *WT1-exon 5* span 2 consecutive exons. The location of the RQ-PCR reaction within one exon may potentially lead to detection of *WT1*-RNA together with *WT1*-DNA, if this is present. Though

all our RNA samples were treated with DNase and in quality controls no DNA contamination of the extracted RNA was ever found, the designed RQ-PCR reaction for KTS detection has been changed – the forward primer moved backward to exon 8. All the tests and *WT1*-KTS detection in normal and patients samples will be repeated with this adjusted method.

In normal developing kidney the four main *WT1* isoforms are present in a ratio 1 (variant A, –/–) : 2.5 (variant B, +/–) : 3.8 (variant C, –/+) : 8.3 (variant D +/+) [Moorwood *et al* 1999, Renshaw *et al* 1997]. In the only study evaluating expression of *WT1* isoforms in normal haematopoietic tissues, Hosen *et al* [Hosen *et al* 2002] found all four *WT1* main spliced variants in CD34+ haematopoietic progenitors, with EX5+/KTS+ variant predominating in CD34+/CD38+ cells. In CD34+/CD38– cells, variable dominant isoforms were identified. We can confirm the presence of all *WT1* isoforms in normal BM cells. Based on our RT-PCR findings of only KTS+ *WT1* variants detected in normal PB on the background of quite low levels of total *WT1* expression in PB cells we can hypothesise that, like in a developing kidney and in agreement with Hosen *et al*, in PB (and possibly other normal haematopoietic tissues), KTS+ variants are present in a higher proportion than KTS–.

Detection and quantification of WT1 expression in diagnostic samples of childhood patients with acute leukaemia and evaluation of WT1 as a potential MRD marker

RT-PCR evaluation of total *WT1* expression was performed only on a limited group of patients' diagnostic samples. They all tested positive already by first round PCR, with variable intensity of the bands.

By RQ-PCR we quantified *WT1* expression in childhood acute leukaemia bone marrow samples (107 B-ALL, 19 T-ALL, 2 AML) and in 3 paired peripheral blood samples (all B-ALL). In childhood B-ALL, we detected a very wide range of *WT1* expression levels but despite that majority of the patients had *WT1* expression similar to normal BM and there was no significant difference in median *WT1* expression between normal BM and childhood B-ALL. The highest detected *WT1* values were up to 2 log above the normal bone marrow *WT1* expression and, on the other side, a proportion of patients showed *WT1* expression below the normal bone marrow *WT1* levels. Contrary to B-ALL, most of childhood T-ALL patients showed *WT1* overexpression with the median significantly higher than in B-ALL patients. The highest *WT1* values in T-ALL were similar to the upper range of *WT1* expression in B-ALL, but no patient expressed *WT1* lower than normal BM levels. One of the AML patients had *WT1* expression quite high, the other within the ranges of normal bone marrow. In the paired BM and PB samples from B-ALL patients, *WT1* expression in PB was slightly lower than in the corresponding BM samples. In addition, *WT1* expression was evaluated in 3 bone marrow samples of childhood patients with other haematological malignancies (NHL, MDS) and the values found were close to those of normal BM.

Comparison of our results with findings of other published studies is complicated to some extent by the different methodology used (with variable sensitivity, different ways of result expression, different amount and type of controls) and heterogeneous patients populations studied (children vs. adults, bone marrow vs. peripheral blood samples, etc.). While it is agreed that *WT1* overexpression, in terms of frequency and *WT1* levels, is lower in ALL than AML and probably lower in children than adults [Menssen *et al* 1995, Inoue *et al* 1997, Niegemann *et al* 1999, Ozgen *et al* 2000, Kreuzer *et al* 2001,

Cilloni *et al* 2002, Trka *et al* 2002, Gaiger *et al* 1999, Kletzel *et al* 2002, Garg *et al* 2003], such a pattern of *WT1* expression we observed has not been described in acute leukaemia before. In the previous studies, *WT1* overexpression above the normal values was found in majority or all of ALL samples [Menssen *et al* 1995, Inoue *et al* 1997, Niegemann *et al* 1999, Ozgen *et al* 2000, Kreuzer *et al* 2001, Cilloni *et al* 2002, Gaiger *et al* 1999, Kletzel *et al* 2002, Garg *et al* 2003, Magyarosy *et al* 2003] and *WT1* expression in acute leukaemia below the ranges of normal controls was never detected. Most of the studies did not distinguish between B and T-ALL; in those analysing these subtypes separately, controversial results were found with higher *WT1* overexpression detected in B-ALL in some of them [Niegemann *et al* 1999] and in T-ALL in others [Ozgen *et al* 2000]. However, these studies investigated *WT1* mostly in adult ALL patients or in heterogeneous groups of children and adults [Menssen *et al* 1995, Inoue *et al* 1997, Kreuzer *et al* 2001, Cilloni *et al* 2002, Garg *et al* 2003], in peripheral blood samples [Kreuzer *et al* 2001, Magyarosy *et al* 2003], using potentially less sensitive PCR techniques of *WT1* detection [Menssen *et al* 1995, Inoue *et al* 1997, Niegemann *et al* 1999, Ozgen *et al* 2000, Gaiger *et al* 1999, Kletzel *et al* 2002, Garg *et al* 2003], and with a limited number or type of normal controls tested [Menssen *et al* 1995, Inoue *et al* 1997, Niegemann *et al* 1999, Ozgen *et al* 2000, Gaiger *et al* 1999]. The highly variable *WT1* levels, including the *WT1* underexpression, we observed in childhood ALL patients at diagnosis, are related only to the leukaemic disease and could not be even partially contributed to individual constitutional variations in *WT1* expression in different patients. This is suggested by the finding that the MRD-negative follow-up samples exhibited very uniform *WT1* expression reflecting normal BM ranges although these patients showed quite variable *WT1* levels in their diagnostic leukaemic samples. As our data also show, the intensity of *WT1* expression in childhood ALL is not linked to the degree of CD34 expression in the leukaemic cells.

Corresponding to other studies [Cilloni *et al* 2002, Garg *et al* 2003] we found *WT1* expression in PB of B-ALL slightly lower than in paired BM samples. We also found, in contrast to the BM samples, median *WT1* expression in B-ALL PB samples over 1 log higher than in normal peripheral blood. This could potentially offer better discrimination between leukaemic and normal samples, but because only few PB samples from patients with high WBC at diagnosis were available to us, this finding cannot be considered significant.

In the cytogenetically defined subgroups of B-ALL, our patients with *MLL-AF4* translocation had extremely high *WT1* levels compared to all the other patients. Patients with *BCR-ABL* p190 translocation also tended to have higher *WT1* expression but it did not reach the statistical significance. These findings are in agreement with results of other authors who described high *WT1* overexpression in ALL with these two translocations [Cilloni *et al* 2002]. We further investigated *WT1* expression in ALL in relation to the two main risk factors – age and leukocyte count. While there was no significant difference in *WT1* levels between patients with WBC in peripheral blood below and over 50 x 10^9 / l, patients of the age between 1 and 10 years had lower *WT1* expression than older patients. Apart from the possible association of *WT1* overexpression with one of the prognostically adverse factors, this finding may also suggest that *WT1* expression in ALL increases with age and explain why the overall *WT1* detected in our childhood patients is lower than the values reported for adult ALL patients. Infants under 1 year also showed high *WT1*

expression but as majority of them expressed *MLL-AF4* translocation, the effect of the very young age on *WT1* expression could not be clearly assessed.

Recent studies have identified *WT1* overexpression as an independent adverse prognostic factor in acute leukaemia associated with an increased risk of relapse [Trka *et al* 2002, Garg *et al* 2003, Barragan *et al* 2004]. Among our childhood ALL patients there was a trend toward higher *WT1* expression in those who relapsed but this did not reach the statistical significance. But interestingly, Kaplan-Meier curves showed that, after dividing patients into three groups with low, normal and high *WT1* levels, patients with *WT1* overexpression together with patients with *WT1* underexpression had a significantly higher risk of relapse (12 and 14 times, respectively) than patients with *WT1* within the physiologic range. This result was supported by univariate analysis of *WT1* expression values showing significantly increased risk of relapse (1.5 / 2-fold) with *WT1* increasing or decreasing from the mean *WT1* expression in childhood ALL. By multivariate analysis, in both cases *WT1* proved to be an independent risk factor. These findings may thus confirm the prognostic impact of *WT1* overexpression in childhood ALL and, at the same time, bring attention to the clinical significance of the abnormally low detected *WT1* levels in some of the childhood ALL patients. Although it is not possible to make a definitive conclusion because of the limited number of relapsed patients with *WT1* underexpression analysed, the phenomenon of *WT1* underexpression should be recognised and its presence and effect in acute leukaemia could be better elucidated by further studies. From the functional point of view, the consequences of *WT1* underexpression may only be hypothesised, as the role of *WT1* in the process of leukaemia development has not yet been sufficiently clarified. It becomes more and more apparent that the regulatory effects of *WT1* on cellular proliferation, differentiation and apoptosis are quite complex, depending on cooperation and interactions of different *WT1* isoforms, a number of other factors and regulators, and the actual status of the cell. In case *WT1* is directly involved in the leukaemogenic pathways, it seems possible that any deviation from normal *WT1* expression and function may lead to disruption of the regulatory networks facilitating leukaemic transformation and/or growth. On the other hand, if changes in *WT1* expression are rather a consequence than a cause of the leukaemic process, *WT1* overexpression may reflect the degree of malignant transformation or proliferation. *WT1* underexpression could then represent a loss of normal *WT1* expression during the progress of the disease or may be a result of leukaemia originating from haematopoietic precursors at the stage of their development when they normally express very low *WT1* levels. Whichever is the case, empirically we know that mutations negatively affecting *WT1* function have been described in acute leukaemia and found to be associated with unfavourable prognosis [Sugiyama *et al* 2001 and 2002, Menssen *et al* 2002], and *WT1* underexpression and mutations altering *WT1* functions are known in Wilms' tumour and other solid malignancies [Sugiyama *et al* 2001, Scharnhorst *et al* 2001, Menssen *et al* 2002, Wagner *et al* 2003, Loeb *et al* 2002].

WT1 overexpression has been successfully used as a marker for detection of minimal residual disease in acute leukaemia, particularly in AML [Kreuzer *et al* 2001, Cilloni *et al* 2002, Trka *et al* 2002, Garg *et al* 2003, Magyarosy *et al* 2003, Ostergaard *et al* 2004]. We investigated the potential of *WT1* for MRD monitoring in childhood ALL. The principle request on a method suitable for MRD detection is its high specificity with sensitivity at least 10^{-3}, ideally 10^{-4} and more. The *WT1* expression we detected in bone marrow samples of childhood ALL patients was very variable and none of the patients had *WT1* at least 2

logs or higher above the background *WT1* expression in the normal bone marrow. Although it has been shown that *WT1* levels can rise before the occurrence of haematological relapse even in bone marrow samples without *WT1* overexpression at diagnosis [Trka *et al* 2002], the question whether monitoring of *WT1* expression in follow-up samples of childhood acute leukaemia patients regardless their *WT1* expression at diagnosis may be useful in predicting haematological relapse has yet to be answered. Also another question, whether detection of *WT1* in peripheral blood rather than in bone marrow may improve the prognostic impact of *WT1* and its use for MRD monitoring, as suggested by some studies [Kreuzer *et al* 2001, Garg *et al* 2003, Magyarosy *et al* 2003], has to be addressed in further trials. Based on the present knowledge, it could be concluded that *WT1* is not a useful marker for MRD monitoring in childhood ALL.

By first round RT-PCR we were able to detect the presence of all four *WT1* variants in a B-ALL sample, and this was confirmed by nested RT-PCR. Using RQ-PCR we quantified expression of exon 5-positive and KTS-positive isoforms in most of the acute leukaemia patients, in whom total *WT1* was previously detected and enough RNA or cDNA left (102 B-ALL, 18 T-ALL, 2 AML). While expression of *WT1-exon 5* isoforms was found to be slightly lower than total *WT1*, expression of *WT1-KTS* isoforms was in most (but not all) patients much higher than total *WT1*, in some cases over 2 logs. At the moment our work aims to exclude that this discrepancy could not be caused by a possible DNA contamination of the RNA samples (see *WT1* isoforms expression in control samples in the previous section).

A recent and so far the only published study on expression of *WT1* isoforms in acute leukaemia patients [Siehl *et al* 2004] detected by RQ-PCR the presence of main *WT1* isoforms in all AML samples with significantly higher expression levels of the exon 5-positive variants. In some other malignancies studied, all *WT1* isoforms were found, either in the same ratio as in the non-malignant corresponding tissue (e.g. Wilms' tumour) [Moorwood *et al* 1999] or in an altered ratio (e.g. breast cancer) [Silberstein *et al* 1997].

6.6. Detection of WT1 protein in haematopoietic tissues by flow cytometry and immunofluorescence microscopy

Based on the availability of anti-WT1 antibodies for detection of WT1 protein in tissues by immunohistochemistry and immunofluorescence microscopy, and on the published data of successful detection of WT1 protein in haematopoietic cells by immunofluorescence microscopy allowing a good discrimination between normal and leukaemic cells according to the WT1 protein expression [Menssen *et al* 1995 and 1997], we have made a considerable effort to establish flow cytometric assay for detection of WT1 protein in haematopoietic cells.

The main problem in the development of this method has been a presence of a background fluorescence in control normal peripheral blood cells, and a relatively low specific fluorescence detectable in WT1 positive K562 cells, which precluded good enough separation and discrimination between WT1 positive and WT1 negative populations. An extensive optimising of the method, that included introduction of directly labelled anti-WT1 antibody, use of a new antibody clone (WLM04) and

a brighter fluorochrome (AlexaFluor488) and optimisation of intracellular staining, could only slightly improve this problem.

The set up method enables measuring levels of WT1 protein in haematopoietic cells by flow cytometry with quantifying WT1 protein expression by percentage of positive cells and their main fluorescence. However, it cannot replace more precise quantification of *WT1* expression by RQ-PCR, and it also does not offer a degree of discrimination between WT1 positive and negative cells that would be sufficient for the application of this method for MRD monitoring purpose.

Together with flow cytometry, we performed detection of WT1 protein in normal peripheral blood, normal bone marrow, acute leukaemia bone marrow samples and K562 cell line by immunofluorescence microscopy. After optimisation, this method gave quite reproducible results. On a low background, strong nuclear staining of WT1 protein was detectable in positive controls (K562 cells) and was not present in normal peripheral blood or bone marrow cells. Interestingly, in addition to this we could find granular perinuclear and cytoplasmic positivity in a proportion of normal bone marrow cells and, in a less pronounced pattern, in normal peripheral blood cells and K562 cells. This positivity seemed to be specific, and in bone marrow and peripheral blood confined more but not exclusively to cells of myeloid lineage.

In the only study evaluating WT1 protein expression in haematopoietic cells by immunoflurescence microscopy [Menssen *et al* 1995 and 1997], granular cytoplasmic fluorescence was detected in mature myelocytic cells with all of several different anti-WT1 antibodies used (monoclonal and polyclonal), but there was no cytoplasmic positivity in mononuclear cells. In agreement with this study we also found granular cytoplasmic positivity predominantly in maturing myeloid cells, but contrary to Menssen we detected this granular positivity in other lineages, too, though to a much lower degree. Our results from immunofluorescence microscopy correlate very well with flow cytometry, where we could detect low levels of WT1 expression in all subsets of peripheral blood cells with relatively higher expression in myeloid population than in B and T-cells. This granular perinuclear and cytoplasmic positivity is also presumably the main cause of the insufficient discrimination between leukaemic and normal cells by flow cytometry analysis.

The nature of the granular perinuclear and cytoplasmic positivity of staining for WT1 protein in normal haematopoietic cells is unclear. The intensity and character of their fluorescence as well as their detection by different clones of antibodies suggest that this is not due to an unspecific binding. The monoclonal antibodies we used (6F-H2 DAKO, WLM04 NeoMarkers) are raised against the N-terminal region of WT1 protein that has little sequence homology to any other known protein. No cross reaction of these antibodies with other zinc finger transcription factors neither other proteins has been described. It therefore seems highly probable that the detected granular perinuclear and cytoplasmic positivity is a result of a presence of WT1 protein in this location. As a transcription factor, WT1 is primarily localised in the nucleus. However, WT1 has been shown to be involved not only in transcription regulation but there is an increasing evidence of its role in posttranscriptional mRNA processing. Later studies proved that WT1 protein undergoes nucleocytoplasmic shuttling and is associated with cytoplasmic ribonucleoprotein particles (RNPs) [Vajjhala *et al* 2003, Niksic *et al* 2004]. Based on their findings we may hypothesise that the granular perinuclear and cytoplasmic positivity we detected is a manifestation of

WT1 protein presence in cytoplasm due to its role in posttranscriptional regulatory processes. In a very recent paper [Nakatsuka *et all* 2006] the authors examined WT1 expression by immunohistochemistry and Western blot analysis in a number of different solid tumours and found diffuse or granular cytoplasmic staining in the majority of positive cases while only few types of tumours showed nuclear staining. More studies will be necessary to elucidate presence, localisations and functions of WT1 and its isoforms in normal and malignant haematopoietic cells.

7. Conclusion and future directions

The ongoing extensive research in immunology, cytogenetics and molecular biology of childhood acute leukaemia, enabled by the development of many modern technologies, has revealed a lot of invaluable information about this malignant disease. Our understanding of the process of leukaemia development and discovering the unique characteristics of leukaemic cells results in practical implications for childhood acute leukaemia management. Research on novel prognostic factors, minimal residual disease markers, as well as therapeutic targets is the base for improvement of present and development of new treatment strategies. So called risk-directed therapy, that has been already successfully implemented in the treatment of childhood ALL, is developing from risk group stratification to a highly individualised approach. New treatment modalities, like antibody therapy, anti-leukaemic vaccines, tyrosine kinase and farnesyl transferase inhibitors, gene therapy and others are tested in clinical trials and offer a lot of hope for the nearest future. Optimisation and standardisation of the new diagnostic techniques and new treatment protocols is necessary for application of the results of scientific and clinical research into standard clinical and laboratory practice. Together, these strategies promise to further improve the outcomes of childhood acute leukaemia therapy, increase the cure rate, minimise side-effects of the therapy, and possibly also offer options for leukaemia prevention.

In this project we focused on the detection of minimal residual disease in childhood acute leukaemia that has been recently proven to be one of the most important prognostic factors in acute leukaemia. All the standard methods of MRD detection – flow cytometric detection of LAPs, PCR detection of chromosomal aberrations, PCR detection of clonal *IG/TCR* rearrangements – were set up, optimised according to international recommendations and latest achievements in the relevant areas, and their characteristics analysed with emphasis on their accuracy, reproducibility and applicability. With these methods we then monitored MRD in a group of 25 patients with childhood acute leukaemia and evaluated (1) the different methods, their performance in practice, correlation of the results and suitability for routine use; (2) the individual MRD levels and leukaemia clearance during the therapy, risk stratification of the patients according the MRD values and the potential clinical impact of the MRD monitoring. Our work could confirm the conclusions of other studies indicating the high quality and reliability of the present methods of MRD detection as well as showing some of their potential pitfalls and limitations. Further studies will be needed (and some of them are ongoing at the moment) to answer the questions about MRD that are still remaining – which type of the sample should be used for MRD monitoring (bone marrow vs. peripheral blood or both); what are the most predictive time-points of MRD detection; how to interpret and compare results of different methods of MRD detection; how to further standardize and simplify the methods of MRD detection employing the latest technologies; and most importantly, whether MRD monitoring in childhood acute leukaemia may improve the prognosis and overall survival of these patients, particularly in prospective risk-adapted strategies.

This study also investigated novel potential prognostic factors and MRD markers. Among many others that have been considered and tested in some studies for this purpose, *WT1* seems to be at present one of the very few which significance is based on results of at least several solid studies, though even in

this case findings of some of them were controversial. Using RT-PCR and RQ-PCR for total *WT1* and its main isoforms we tried to elucidate the *WT1* expression patterns and its clinical implication in childhood acute leukaemia, particularly ALL, where *WT1* has been least studied and its role remained unclear. On a representative group of childhood ALL patients we could show that *WT1* expression in this type of leukaemia is much lower than that reported in AML or adult ALL and that *WT1* therefore will not be a suitable MRD marker in these patients. The prognostic value of *WT1* overexpression and also underexpression, which we found in a proportion of patients and which has not been reported previously, will require confirmation in further studies. At present we focus on the expression analysis of the main *WT1* isoforms in normal haematopoietic cells and childhood acute leukaemia patients and their relation to the other biological features of the disease and its clinical course. This part of the study is ongoing. In the future we would also like to evaluate *WT1* expression in paired peripheral blood and bone marrow samples and address the question of possible advantages of *WT1* monitoring in peripheral blood.

We would be happy if this work may contribute to the advances in management of childhood acute leukaemia and bring benefit to the childhood acute leukaemia patients.

8. Protocols and reagents

Protocol 1: Defrosting cells and starting initial cell cultures

Work under strictly sterile conditions in a class II laminar flow cabinet for cell line cultures.

1. Pre-chill RPMI medium (RPMI 1640 with Glutamax) to 4 °C, pre-warm 20 % complete RPMI medium (RPMI 1640 with Glutamax + 20 % FBS + P/S) to 37 °C
2. Pour 45 ml of pre-chilled RPMI medium into 50 ml sterile tube(s)
3. Place the ampoule with the frozen cells onto ice, with a Pasteur pipette add a bit of RPMI medium from the 50 ml sterile tube into the ampoule with the cells, wash by gentle pipetting up and down few times, then aspirate the liquid and transfer to the RPMI medium in the 50 ml tube; repeat until the whole contents of the ampoule is defrosted and transferred to the RPMI in the 50 ml tube
4. Spin down at 400 g (1400 rpm) for 5 min, discard the supernatant
5. Resuspend the pellet of cells with 6 - 10 ml of pre-warmed 20 % complete RPMI medium
6. Transfer into 25 ccm culture flasks with perforated lids
7. Put into the incubator and grow at 37°C and 5 % CO_2 for 2 - 4 days (eventually more depending on the cell type)
8. Check regularly the cellular density of the culture under the microscope and when it reaches the appropriate level, do the first passaging

10 % or 20 % complete RPMI medium

	10 %	20 %
RPMI 1640 with Glutamax	450 ml	400 ml
Penicillin/Streptomycin (10 000 U/10 mg/ml)	5 ml	5 ml
FBS (Foetal bovine serum)	50 ml	100 ml
filter sterilise (0.2 µm filter)		

Protocol 2: Passaging cells in cell cultures

A. Passaging cells in suspension

Work under strictly sterile conditions in a class II laminar flow cabinet for cell line cultures.

1. Pre-warm 10 % or 20 % complete RPMI medium (depending on the cell type) to 37 °C
2. Transfer the contents of a flask with the cell suspension into a sterile tube
3. Spin down at 400 g (1400 rpm) for 5 min, discard the supernatant
4. Resuspend the pellet of cells with an appropriate amount of pre-warmed 10 % or 20 % complete RPMI medium (depending on the pellet size, about 2 - 4 ml per 1 x 10^7 cells)
5. Put 16 - 18 ml of pre-warmed 10 % or 20 % complete RPMI medium into 75 ccm culture flask(s) with perforated lids (or 6 - 8 ml into 25 ccm flasks)
6. Transfer 2 - 4 ml of the cell suspension into the flask(s) (about 1 x 10^7 cells into a 75 ccm flask to get a total volume of 20 ml, and about 0.5 x 10^7 cells into a 25 ccm flask to get a total volume of 8 - 10 ml)
7. Put into the incubator and grow at 37°C and 5 % CO_2 for 2 - 4 days (depending on the cell type)
8. Check regularly the cellular density of the culture under the microscope and repeat the passaging when appropriate density is reached

B. Passasging cells in monolayer

Work under strictly sterile conditions in a class II laminar flow cabinet for cell line cultures.

1. Remove the contents of the flask and if it contains cells in suspension, process according the protocol 2A.
2. Add trypsin, 1 - 1.5 ml for 25 ccm flask or 2.5 - 3 ml for 75 ccm flask, incubate at 37°C for 5 min
3. Add HBSS medium to the total volume of 5 ml per flask
4. Scrape the flask surface with a sterile scraper
5. Add 5 ml of HBSS and transfer the whole contents into a sterile tube
6. Spin down at 400 g (1400 rpm) for 5 min, discard the supernatant
7. Go on as in case of cell suspension (protocol 2A, step 4.)

<u>Cell lines requiring 10 % complete RPMI medium:</u>
HL-60, MOLT-3, SD-1, K-562, BV-173, KY-01, 697, REH, CCRF-CEM, MOLT-16, NB-4, KASUMI-1

<u>Cell lines requiring 20 % complete RPMI medium:</u>
MV4-11, ME-1

Protocol 3: Freezing cells

Work under strictly sterile conditions in a class II laminar flow cabinet for cell line cultures.

1. Transfer the contents of a flask with the cell suspension into a sterile tube
2. Centrifuge at 400 g (1400 rpm) for 5 min, discard the supernatant
3. Resuspend the pellet of cells with an appropriate amount of pre-warmed 10 % or 20 % complete RPMI medium (depending on the pellet size, about 2 - 4 ml per 1 x 10^7 cells)
4. Count the cells and adjust their concentration to 5 - 10 x 10^6 cells / ml
5. Put 3 - 5 ml of the suspension into a new sterile tube and place on ice
6. Pre-label 2 ml cryo-vials (6 - 10) and place on ice
7. Prepare 2X freezing medium and place on ice
8. Add slowly, drop-wise with permanent mixing, the same volume of 2X freezing medium to the cell suspension
9. Aliquot into 2 ml cryo-vials on ice, 1 ml per tube
10. Put in a polystyrene box and freeze at - 70 °C, leave for one day
11. Transfer into liquid nitrogen (- 196 °C)

Remark: This protocol can be also used for freezing bone marrow or peripheral blood cells, after mononuclear separation and washing.

Freezing medium 2X

RPMI 1640 with Glutamax	4 ml (40 %)
FBS	4 ml (40 %)
DMSO (dimethylsulfoxide, cell culture grade)	2 ml (20 %)

filter sterilise (0.2 µm filter)

Protocol 4: Counting cells

1. Prepare the cell suspension
2. Mix 10 µl of the cell suspension with either 20 µl (dilution factor 3) or 90 µl (dilution factor 10) of ethidium bromide-acridine orange solution
3. Place a cover glass on a haemocytometer and load the chamber with the cell/EBAO mix (about 10 µl)
4. Place the haemocytometer under fluorescent microscope using green fluorescent light, and count all the cells of interest (green cells - alive, orange cells - dead) in the chamber (in the central 25 squares)
5. Calculate the concentration of cells in the suspension:
   concentration [cells x 10^6 / ml] = (number of cells in the chamber x dilution factor) / 100

EBAO solution

Ethidium Bromide	20 mg
Acridine Orange	4 mg
D-PBS	1000 ml

(AO binds to RNA of vital cells and stains them green, ethidium bromide binds to DNA of death cells and stains them orange)

Protocol 5: Mononuclear cell separation

1. If the bone marrow is not previously diluted in RPMI medium, dilute the bone marrow, peripheral blood or peripheral stem cell sample in D-PBS up to the total volume of 10 – 12 ml
2. Pour Lymphoprep (of room temperature, 20 °C) into a sterile 20 ml tube, in a half of the volume of the sample (5 – 6 ml)
3. With a Pasteur pipette, carefully layer the diluted sample over Lymphoprep
4. Centrifuge at 1800 rpm for 25 min
5. Remove the mononuclear-rich interphase by a Pasteur pipette, put into a new sterile 20 ml tube and add D-PBS up to 20 ml
6. Centrifuge at 1800 rpm for 10 min, discard the supernatant
7. Resuspend the cells in 0.5 – 5 ml of the appropriate solution (D-PBS for RNA and DNA extraction, CellWash/FBS5% for FC), count the cells and adjust the concentration as required (usually 1 x 10^7 / ml)
8. Keep the cell suspension on ice an immediately proceed according to the further methods

Protocol 6: RNA extraction
- using RNeasy Mini Kit QIAGEN

Work at room temperature, in a laminar flow cabinet for RNA work.

1. Resuspend the cells (separated mononuclear cells or cell line) in PBS at concentration 1 x 10^7 / ml
2. Pipette the cell suspension into pre-labelled 1.5 ml microfuge tube(s), up to 1 x 10^7 cells per tube
3. Centrifuge at 8 000 rpm for 1 min, aspirate the supernatant and vortex the cell pellet
4. Add 600 µl of RLT buffer to prepare cell lysate, vortex or pipette up and down
5. Store at - 80 °C or proceed with next steps
6. Pipette the whole contents of the microfuge tube onto a QIAshredder Spin Column placed in a 2 ml collection tube
7. Centrifuge at 13 000 rpm for 2 min to homogenise the sample, discard the column
8. Add 1 volume (600 µl) of 70 % ethanol to the homogenised lysate in the collection tube and mix well by pipetting
9. Apply 650 µl of the sample including any precipitate to an RNeasy Mini Column placed in a 2 ml collection tube; cover the collection tube with the rest of the sample with a foil
10. Centrifuge at 10 000 rpm for 30 sec, discard the flow-through
11. Apply the rest of the sample to the RNeasy Mini Column
12. Centrifuge again at 10 000 rpm for 30 sec, discard the flow-through
13. Pipette 350 µl of RW1 buffer onto the RNeasy Mini Column
14. Centrifuge at 10 000 rpm for 30 sec, discard the flow-through
15. Prepare DNase solution: resuspend the lyophilised DNase I in 550 µl of sdH_2O, gently mix; add 10 µl of the resuspended DNase I to 70 µl of RDD buffer and gently mix to get the volume of 80 µl per tube
16. Add 80 µl of the DNase I solution directly onto the membrane of RNeasy Mini Column, incubate at room temperature for 15 min
17. Pipette 350 µl of RW1 buffer onto the RNeasy Mini Column
18. Centrifuge at 10 000 rpm for 15 sec, discard the flow-through
19. Pipette 500 µl of RPE buffer onto the RNeasy Mini Column
20. Centrifuge at 10 000 rpm for 15 sec, discard the flow-through
21. Pipette 500 µl of RPE buffer onto the RNeasy Mini Column
22. Centrifuge at 13 000 rpm for 2 min to dry the RNeasy membrane, discard the flow-through and the collection tube
23. Place the column into a new 2 ml collection tube
24. Centrifuge at 13 000 rpm for 1 min
25. Discard the collection tube, transfer the RNeasy Mini Column into a new pre-labelled 1.5 ml microcentrifuge tube
26. Pipette 50 µl of RNase-free water directly onto the RNeasy membrane, incubate for 1 min at room temperature
27. Centrifuge at 10 000 rpm for 1 min to elute RNA
28. Repeat step 26. and 27. if more than 30 µg of RNA is expected or if necessary to increase the amount of extracted RNA
29. Store RNA at - 80 °C, event. before it take 1 - 5 µl of RNA to check its purity and integrity
30. Check the purity and integrity of the extracted RNA by gel electrophoresis: run the extracted RNA diluted in sdH_2O (5 µl of RNA + 5 µl of sdH_2O or 1 µl of RNA + 9 µl sdH_2O) with 2 µl of 6X load dye on 2 % agarose gel together with 100 b DNA ladder
    - 2 sharp bands corresponding to 18S and 28S rRNA (which represents over 90 % of total RNA) should be present; a presence of contaminating DNA would appear as another band of a very large size

Protocol 7: DNA extraction
- using QIAamp DNA Blood Mini Kit QIAGEN

Work at room temperature, in a laminar flow cabinet for DNA work.

1. Resuspend the cells (separated mononuclears or cell line) in PBS at concentration 1 x 10^7 / ml
2. Pipette the cell suspension into pre-labelled 1.5 ml microfuge tube(s), up to 1 x 10^7 cells per tube
3. Centrifuge at 8 000 rpm for 1 min, aspirate the supernatant and vortex the cell pellet
4. Add 250 µl of PBS, vortex (carrier DNA can be used in case of a very small amount of DNA)
5. Add 250 µl of AL buffer, vortex, event. briefly centrifuge
6. Store at - 80 °C or proceed with next steps
7. Prepare protease/proteinase K solution: resuspend the lyophilised enzyme in 1.2 ml (50 preparations) or 5.5 ml (250 preparations) of protease/protienase K solvent, mix gently by pipetting, aliquot adequate volumes to sterile tubes and store at - 20 °C (protease can be stored at 4 °C for up to 2 months)
8. Add 25 µl of protease or proteinase K, vortex for 15 sec, and incubate at 56 °C for 10 min, then briefly centrifuge to remove drops from the inside of the lid
9. Add 250 µl of 100 % ethanol, vortex for 15 sec and briefly centrifuge
10. Apply the sample (750 µl) to the QIAamp Spin Mini Column placed in a 2 ml collection tube
11. Centrifuge at 8 000 rpm for 1 min, discard the filtrate
12. Pipette 500 µl of AW1 buffer onto the column
13. Centrifuge at 8 000 rpm for 1 min, discard the filtrate
14. Pipette 500 µl of AW2 buffer onto the column
15. Centrifuge at 13 000 rpm for 3 min, discard the filtrate and the collection tube
16. Place the column into a new 2 ml collection tube
17. Centrifuge at 13 000 rpm for 1 min
18. Discard the collection tube, transfer the QIAamp Spin Mini Column into a new pre-labelled 1.5 ml microcentrifuge tube
19. Pipette 200 µl of AE buffer onto the column, incubate for 1- 5 min at room temperature
20. Centrifuge at 8 000 rpm for 1 min to elute DNA
21. Repeat step 19. and 20. if necessary to increase the amount of extracted DNA
22. Store DNA at - 80 °C (or - 20 °C)

for clonality assays prepare 20 – 50 µl of DNA in concentration 0.1 µg (100 ng) / µl:
- centrifuge in a heated vacuum centrifuge at medium heat for 10 – 30 min to concentrate the DNA
- add sdH_2O to dilute the DNA

Protocol 8: Titration of antibodies for FC

Work at room temperature, switch off lights

1. Use a suspension of cells expressing the antigen detected by the antibody to be titrate (whole blood or bone marrow or separated mononuclear cells) diluted in CellWash/FCS5% or PBS/BSA/SA at concentration of WBC 1 x 10^7 / ml
2. Prepare and label 9 Falcon 5ml polystyrene tubes

Tube	1.	2.	3.	4.	5.	6.	7.	8.	9.
Label	Neg. Ctrl.	Isotype Ctrl.	Ab 40 µl	Ab 20 µl	Ab 10 µl	Ab 5 µl	Ab 2.5 µl	Ab 1.25 µl	Ab 0.625 µl

3. Add 40 µl of CellWash/FCS5% into tubes 4. – 9.
4. Add isotype control in the amount of protein equal to that of 5 µl of the titrated antibody into tube 2.
5. Add 40 µl of undiluted antibody into tube 3
6. Add 40 µl of undiluted antibody to tube 4, vortex, exchange the pipette tip
7. Take 40 µl of the contents of tube 4 and put to tube 5, vortex, exchange the pipette tip
8. Take 40 µl of the contents of tube 5 and put to tube 6, vortex, exchange the pipette tip
9. Take 40 µl of the contents of tube 6 and put to tube 7, vortex, exchange the pipette tip
10. Take 40 µl of the contents of tube 7 and put to tube 8, vortex, exchange the pipette tip
11. Take 40 µl of the contents of tube 8 and put to tube 9, vortex
12. Take 40 µl of the contents of tube 9 and discard
13. Add the cell suspension to all tubes (1 x 10^6 cells per tube) and proceed with the staining according to the protocol for surface, eventually intracellular, antigen staining
14. Acquire the samples on flow cytometer and carry out the analysis according to the protocols
15. Determine the mean fluorescence for each dilution/amount of the antibody
16. Enter the data into Microsoft Excel and create the titration curve
17. Chose the optimal dilution/amount of the antibody as that one closest to but still below the maximum intensity plateau

PBS/BSA(1%)/SA(0.1%) solution

Bovine Serum Albumin	5 g
Sodium Azide	0.5 g
D-PBS	500 ml

filter sterilise (0.2 µm filter)

Protocol 9: FC staining for surface antigens – method of whole blood lysis

1. Dilute whole bone marrow or peripheral blood sample or separated mononuclear cells in CellWash/FBS5% to the WBC concentration of 1 x 10^7 / ml
2. Prepare and label Falcon 5 ml polystyrene tubes
3. Add monoclonal antibodies to each of the tubes according to the listed panels for B-ALL, T-ALL or AML respectively
4. Add 100 µl of the sample to each tube, vortex
5. Incubate at room temperature in the dark for 12 min
6. Add 2 ml of 1X FACS Lysing solution (diluted 1:10 in sdH_2O), vortex
7. Incubate at room temperature in the dark for another 10 min
8. Centrifuge at 400 g for 5 min, discard the supernatant
9. Add 2 ml of CellWash, vortex
10. Centrifuge at 400 g for 5 min, discard the supernatant
11. Add 0.5 ml of CellFix (diluted 1:10 in sdH_2O), vortex
12. Keep in the dark
13. Acquire immediately or keep at 4°C in the dark until acquisition, acquire within 8 - 12 hours

CellWash/FBS 5%

CellWash (BD)	475 ml
Foetal Bovine Serum	25 ml
total	500 ml

Protocol 10: FC staining for intracellular antigens
- using IntraStain DAKO kit

1. Dilute whole bone marrow or peripheral blood sample or separated mononuclear cells in CellWash/FBS to the WBC concentration of 1 x 10^7 / ml
2. Prepare and label Falcon 5 ml polystyrene tubes
3. Add monoclonal antibodies against surface markers to each of the tubes according to the listed panels for B-ALL or T-ALL
4. Add 100 µl of the sample to each tube, vortex
5. Incubate at room temperature in the dark for 12 min
6. Add 2 ml of CellWash, vortex
7. Centrifuge at 400 g for 5 min, discard the supernatant, vortex the pellet
8. Add 100 µl of IntraStain (DAKO) Reagent A – Fixation , vortex
9. Incubate at room temperature in the dark for 15 min
10. Add 2 ml of CellWash, vortex
11. Centrifuge at 400 g for 5 min, discard the supernatant, vortex the pellet
12. Add monoclonal antibodies against intracellular markers to each of the tubes according to the listed panels for B-ALL or T-ALL
13. Add 100 µl of IntraStain (DAKO) Reagent B – Permeabilisation , vortex
14. Incubate at room temperature in the dark for 15 min
15. Add 2 ml of CellWash, vortex
16. Centrifuge at 400 g for 5 min, discard the supernatant
17. Add 0.5 ml of CellFix (diluted 1:10 in sdH_2O), vortex
18. Keep in the dark
19. Acquire immediately or keep at 4°C in the dark until acquisition, acquire within 8 hours

Protocol 11: Determination of concentration and purity of RNA and DNA
- by spectrophotometry (SPEC)

1. Work with undiluted RNA/DNA or prepare appropriate dilutions in sdH_2O (RNase-free, DNase-free) depending on the type of the spectrophotometer
2. Blank the spectrophotometer with sdH_2O
3. Measure the absorbance at 260 nm (A_{260}), take 2 - 3 measurements and use the average
4. Calculate the concentrations:
   RNA c [µg / ml] = A_{260} x 40 x dilution factor (an absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml)
   DNA c [µg / ml] = A_{260} x 50 x dilution factor (an absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml)
5. The purity of RNA/DNA may also be estimated by calculating the ratio of absorbance at 260 nm (corresponds to RNA/DNA) and 280 nm (corresponds to protein contaminants)
   pure RNA A_{260} / A_{280} = 1.9 - 2.1 (at pH 7.5)
   pure DNA A_{260} / A_{280} = 1.7 - 1.9 (at pH 7.5)
6. To adjust the RNA/DNA concentration to that required:
   - dilute by adding sdH_2O
   - concentrate by centrifuging the samples in heated vacuum centrifuge (cover the microfuge tubes with RNA/DNA samples with pierced lids and centrifuge at medium heat for 10 – 20 min)

Protocol 12: RT reaction
- according to EAC protocols

Work in a laminar flow cabinet for RNA work.
Thaw all the tubes (reagents, controls, pts' samples), gently vortex, briefly spin down, keep on ice.

RNA	1 µg in 10.5 µl of sdH_2O (RNase/DNase free, molecular biology grade)

I. RNA denaturation:
incubation: 70°C for 10 min
4°C for 5 min
quick chill on ice

Random primers (1000 µM)	0.5 µl (25 µM)
dNTPs (10 mM)	2 µl (1 mM)
5X First strand buffer	4 µl (50 mM/ 75 mM/ 3 mM)
DTT (100 mM)	2 µl (10 mM)
RNasin (40 U / µl)	0.5 µl (= 20 U)
SuperScript II (200 U / µl) (MMLV-RT)	0.5 µl (= 100 U)
Total volume	20 µl

II. RT:
gently vortex, ev. spin down
incubation: 25°C for 10 min (random primers activation)
42°C for 45 – 50 min
99°C for 3 min (inactivation)
4°C at the end

(all indicated concentrations are for the final volume of the reaction)

For RQ-PCR then add 30 µl of sd H_2O to the product of RT.

Protocol 13: RT-PCR, first round and nested
- according to BIOMED-1 protocols

Work in a laminar flow cabinet for DNA work.
Set the reactions in singlicate for:
- all the samples
- positive control (positive cell line or pt), negative control (negative cell line or pt) and no template control (sdH_2O).

Thaw all the tubes (reagents, controls, pts' samples), gently vortex, briefly spin down, keep on ice.

cDNA	2 µl (10 % of RT reaction)
or	
DNA	1 µl (2 % of first round PCR reaction)
Fwd-primer (20 µM; 20 pmol/µl)	1 µl (400 nM)
Rvs-primer (20 µM; 20 pmol/µl)	1 µl (400 nM)
dU/dNTPs (10 mM)	1 µl (200 µM)
10X PCR Gold buffer	5 µl (15 mM/ 50 mM)
$MgCl_2$ (25 mM)	2 – 8 µl (2 µl ≅ 1 mM) - acc. to the optimisation
(DMSO 100%	1 – 2.5 µl (1 µl ≅ 2 %) - acc. to the optimisation
Uracil-N-Glycosylase (1 U / µl)	0.5 µl (0.5 U) - in the first round PCR only!
AmpliTaq Gold DNA polymerase (5 U / µl)	0.25 µl (1.25 U)
sdH_2O	to 50 µl
gently vortex, ev. spin down	

incubation:	37°C for 2 min	(UNG step)
	95°C for 10 min	(DNA polymerase activation)
	35 cycles: 94°C for 30 sec	(denaturation)
	65°C for 1 min	(annelation)
	72°C for 1 min	(extension)
	16°C at the end	

(all indicated concentrations are for the final volume of the reaction)

Protocol 14: Analysis of RT-PCR products by electrophoresis on agarose gel

1. Prepare 2 % agarose gel: dissolve 2 g of ultrapure agarose in 100 ml 1X TAE buffer (Tris/acetate/EDTA), microwave until melted, cool down, add Ethidium Bromide (solution 10 mg / ml) 5 µl per 100 ml of gel (0.5 µg / ml), pour into the tray, insert comb(s) and leave to solidify
2. Pipette 2 µl of 6X Loading dye/buffer into pre-labelled 1.5 ml microfuge tube(s) and add 10 µl of the PCR product
3. Put the tray with the agarose gel into electrophoretic tank and fill with 1X TAE buffer up to the marked line, take out the comb(s)
4. Pipette the samples into the wells in the gel, add 12 µl of size marker (100 bp DNA Ladder) on both sides
5. Close the lid, connect electrodes (as negatively charged, DNA and RNA run to anoda), and run the samples on the gel at 80 – 120 V for 30 – 60 min
6. Switch off the source, disconnect the electrodes, take out the gel and examine under UV light, take a photograph

Protocol 15: $MgCl_2$ titration for RT-PCR

1. Perform RT reaction with cell line RNA containing the target chromosomal aberration and its 10^{-1} and 10^{-2} dilutions in cell line RNA negative for this aberration; use the negative cell line RNA and sdH_2O as negative controls
2. Prepare PCR master mixes with $MgCl_2$ concentrations 0 – 6 mM for each set of the primers
3. Set the PCR reactions with the prepared cDNA in duplicates and perform PCR in the standard way
4. Run the PCR products on agarose gel and analyse the quality of the bands obtained by different reactions
5. Chose the $MgCl_2$ concentration giving the strongest and clearest bands
6. If the results are not satisfactory even with the optimal $MgCl_2$ concentration, consider addition of DMSO in concentrations 1 – 5 % - optimise by titration in the same way

Protocol 16: RQ-PCR for detection of chromosomal aberrations and WT1 expression - according to EAC protocols

Work in a laminar flow cabinet for DNA work.
Set the reactions in triplicate for the target gene and duplicate for the control gene and standards/calibrators, for:
- all the samples
- positive control (positive cell line or pt or standards/calibrators), negative control (negative cell line or pt) and no template control (sdH_2O).

Thaw all the tubes (reagents, controls, pts' samples), gently vortex, briefly spin down, keep on ice.

cDNA	5 µl (10 % of the diluted RT reaction, 100 ng RNA equivalent)	
Fwd-primer (15 µM; 15 pmol/µl)	0.5 µl (300 nM)	or 20X B2M PDAR 1.25 µl
Rvs-primer (15 µM; 15 pmol/µl)	0.5 µl (300 nM)	(Vic-labelled)
Probe (5 µM; 5 pmol/µl)	1 µl (200 nM)	
	(0.5 µl (100 nM) in case of AML1-ETO)	
2X TaqMan Universal Master Mix	12.5 µl	
sdH_2O	to 25 µl	
gently vortex, ev. spin down		

incubation:	50°C for 2 min	(UNG step)
	95°C for 10 min	(DNA polymerase activation)
	50 cycles: 95°C for 15 sec	(denaturation)
	60°C for 1 min	(annelation/extension)

(all indicated concentrations are for the final volume of the reaction)

data analysis: threshold set at 0.1, except for PML-RARA 0.05
baseline set at 3 – 15 cycles, except for B2M 3 – 10 cycles

Protocol 17: RNA dilution series for RT-PCR and RQ-PCR sensitivity tests

Work in a laminar flow cabinet for RNA work.

1. Prepare a positive RNA (containing the target gene) and a control negative RNA (without expression of this gene), leave both to thaw completely on ice, measure the RNA concentration of both by SPEC and calculate the volume containing 1 µg of RNA
2. Pre-label 6 sterile microfuge 1.5 ml tubes, add 45 µl of sdH_2O to each of them
3. Add 5 µl of the positive RNA to tube 1, gently vortex, leave for 5 – 10 min at room temperature, exchange the pipette tip (dilution 10^{-1})
4. Take 5 µl of the contents of tube 1 and put to tube 2, gently vortex, leave for 5 – 10 min at room temperature, exchange the pipette tip (dilution 10^{-2})
5. Take 5 µl of the contents of tube 2 and put to tube 3, gently vortex, leave for 5 – 10 min at room temperature, exchange the pipette tip (dilution 10^{-3})
6. Take 5 µl of the contents of tube 3 and put to tube 4, gently vortex, leave for 5 – 10 min at room temperature, exchange the pipette tip (dilution 10^{-4})
7. Take 5 µl of the contents of tube 4 and put to tube 5, gently vortex, leave for 5 – 10 min at room temperature, exchange the pipette tip (dilution 10^{-5})
8. Take 5 µl of the contents of tube 5 and put to tube 6, gently vortex, leave for 5 – 10 min at room temperature (dilution 10^{-6})
9. Put 1 µg of undiluted positive RNA and the same volume of each of the 6 dilutions into corresponding pre-labelled tubes for PCR reaction
10. Add control negative RNA into the tubes of the dilution series: 0.9 µg into 10^{-1} dilution, 0.99 µg into 10^{-2} dilution, and 1µg into 10^{-3} to 10^{-6} dilutions
11. Add sdH_2O to the PCR tubes up to the total volume of 10.5 µl
12. Perform RT reaction according to the protocol

Protocol 18: PCR – multiplex clonality assay
- according to BIOMED-2 and InVivoScribe protocols

Work in a laminar flow cabinet for DNA work.
Set the reactions in singlicate for:
1) template amplification control (Specimen Control Size Ladder) for each sample
   - to check that no inhibitors of amplification (eg. heparin) and DNA of sufficient quality and quantity are present
   - either prior or together with the clonality screening.
2) positive control (positive DNA), negative control (human polyclonal genomic DNA) and no template control (sdH_2O) for each of the reactions.

Thaw all the tubes (master mixes, controls, pts' samples), gently vortex, briefly spin down, keep on ice.

DNA 100 (– 400) ng / µl in sdH_2O or TE (10 mM Tris-HCl + 1 mM EDTA) 2.5 µl

IG or TCR or Specimen Control Size Ladder master mix (multiplex tubes)	22.5 µl
Uracil-N-Glycosylase (1 U / µl)	0.25 µl (0.25 U)
AmpliTaq Gold DNA polymerase (5 U / µl)	0.125 µl (0.625 U)

gently vortex, ev. spin down

incubation:	37°C for 2 min	(UNG step)
	95°C for 7 min	(DNA polymerase activation)
	35 cycles: 95°C for 45 sec	(denaturation)
	60°C for 45 sec	(annelation)
	72°C for 90 sec	(extension)
	72°C for 10 min	(final extension)
	16°C at the end	

(use the "calculated" option for temperature measurement with the MJ Research PTC-200 thermocycler or others)

Multiplex clonality assays used for screening:
B-ALL: 1. IGH VH-JH-FR1 (IGH tube A)
IGK Vκ-Kde (IGK tube B)
IGH DH-JH and DH7-JH (IGH tube D and E)
2. IGH VH-JH-FR2 and 3 (IGH tube B and C)
IGK Vκ- Jκ (IGK tube A)
3. TCRG Vγ1f+ Vγ10+ Jγ and Vγ9+ Vγ11+ Jγ (TCRG tube A and B)
TCRD (TCRD tube)

T-ALL: 1. TCRD (TCRD tube)
2. TCRG Vγ1f+ Vγ10+ Jγ and Vγ9+ Vγ11+ Jγ (TCRG tube A and B)
3. TCRB Vβ +Jβ1, Vβ +Jβ2, Dβ +Jβ (TCRB tube A, B and C)

Protocol 19: Analysis of PCR- clonality products by electrophoresis on MetaPhor agarose gel

1. Prepare 2 % MetaPhor agarose gel: dissolve 2 g of MetaPhor agarose in 100 ml 1X TBE buffer (Tris/borate/EDTA), slowly microwave until melted, cool down, add Ethidium Bromide (solution 10 mg / ml) 5 µl per 100 ml of gel (0.5 µg / ml), pour into the casting tray, insert a comb, leave to cool down and then place into a fridge for 1 – 1.5 hours to solidify
2. Pipette 2 µl of 6X Loading dye/buffer into pre-labelled 1.5 ml microfuge tube(s) and add 10 µl of the PCR product
3. Put the tray with the agarose gel into electrophoretic tank and fill with 1X TBE buffer up to the marked line, take out the comb
4. Load the samples into the wells in the gel, add 12 µl of a size marker (100 bp DNA Ladder) on both sides
5. Close the lid, connect electrodes (as negatively charged, DNA and RNA run to anoda), and run the samples on the gel at 80 V for 60 – 90 min (in a cold room)
6. Switch off the source, disconnect the electrodes, take out the gel and examine under UV light, photograph the gel

Interpretation of the results:
One or two prominent bands within the valid size range indicate the presence of monoclonal population (two bands may be produced in cases when the initial rearrangement was non-productive and was followed by rearrangement of the other homologous chromosome).
If only a single band is present, proceed directly to further steps - cleaning and sequencing the PCR product.
If 2 or more bands are present, excise the bands from the gel, clean up and sequence separately, or repeat in singleplex.
If the band(s) are not strong enough (not enough products for sequencing) either repeat the reaction with other multiplex tube to get stronger band(s) (eg. in case of IHG-FR1 repeat with IHG-FR2 and 3), or excise the band(s), clean up, and repeat the PCR reaction with the DNA obtained from a single band.

Protocol 20: Analysis of PCR- clonality products by GeneScan (ABI fluorescence detection system)

1. Pre-label 0.5 ml microcentrifuge tubes
2. Add 10 µl of Hi-Di Formamide containing ROX size standards to each of the tubes (use GeneScan-400 ROX Size Standard 0.5 µl added to 9.5 µl of Hi-Di Formamide)
3. Add 1 µl of the PCR reaction products to the corresponding tubes, mix well
4. Pipette the content of the tubes on an optical plate, add Hi-Di Formamide to fill a multiple of 16 wells
5. Put the plate to a PCR machine, heat to 95°C for 2 min, then quick chill on ice for 5 min
6. Cover the plate with rubber cover, put into the ABI 3100 machine (capillary electrophoresis fluorescence detection instrument) and run
7. Analyse the results

Protocol 21: PCR – singleplex clonality assay
- according to BIOMED-2

Work in a laminar flow cabinet for DNA work.
Set the reactions in singlicate for:
- all samples
- positive control (positive DNA), negative control (human polyclonal genomic DNA) and no template control (sdH_2O).

Thaw all the tubes (reagents, controls, pts' samples), gently vortex, briefly spin down, keep on ice.
Use the primers according to the BIOMED-2 protocols.

DNA 100 (– 400) ng / µl in sdH_2O or TE (10 mM Tris-HCl + 1 mM EDTA) 2.5 µl

Fwd-primer (10 pmol / µl)	1 µl (10 pmol)	or 0.5 µl
Rvs-primer (10 pmol / µl)	1 µl (10 pmol)	or 0.5 µl
dU/dNTPs (10 mM)	1 µl (200 µM)	or 0.5 µl
10X PCR Gold buffer	5 µl (15 mM/ 50 mM)	or 2.5 µl
(or GeneAmp 10X PCR Buffer II – for TCRB)		
$MgCl_2$ (25 mM) (according to the optimisation)	3 µl (2 – 6 µl)	or 1.5 µl
(2 µl ≅ 1 mM)	4 µl in case of TCRB or TCRD	
Uracil-N-Glycosylase (1 U / µl)	0.5 µl (0.5 U)	or 0.25 µl
AmpliTaq Gold DNA polymerase (5 U / µl)	0.25 µl (1.25 U)	or 0.125 µl
sdH_2O	to 50 µl	or 25 µl
gently vortex, ev. spin down		

incubation:	37°C for 2 min	(UNG step)
	95°C for 7 min	(DNA polymerase activation)
	35 cycles: 95°C for 45 sec	(denaturation)
	60°C for 45 sec	(annelation)
	72°C for 90 sec	(extension)
	72°C for 10 min	(final extension)
	16°C at the end	

(use the "calculated" option for temperature measurement with the MJ Research PTC-200 thermocycler or others)

Protocol 22: Purification of PCR products
- using QIAquick PCR Purification Kit QIAGEN

- to purify single- or double-stranded DNA of the size 100 bp – 10 kb from PCR

Work in a laminar flow cabinet for DNA work.
1. Add 5 volumes of PB buffer to 1 volume of the PCR sample, vortex, briefly spin down
2. Apply the sample to a QIAquick column placed in a 2 ml collection tube
3. Centrifuge at 13 000 rpm for 1 min, discard the flow
4. Add 750 µl of PE buffer to the QIAquick column
5. Centrifuge at 13 000 rpm for 1 min, discard the flow and the collection tube
6. Place the QIAquick column into a new 2 ml collection tube
7. Centrifuge at 13 000 rpm for 1 min, discard the flow and the collection tube
8. Place the QIAquick column into a clean pre-labelled 1.5 ml microcentrifuge tube
9. Add 50 µl (30 – 50 µl) of EB buffer or sdH_2O directly onto the centre of QIAquick column membrane, allow to stand for 1 min
10. Centrifuge at 13 000 rpm for 1 min to elute DNA, discard the column
11. Store the DNA at - 20 °C or - 80 °C

Protocol 23: Extraction and cleaning of PCR products from agarose gel
- using QIAquick Gel Extraction Kit QIAGEN

- for extraction and purification of DNA of the size 70 bp – 10 kb from standard or low-melt agarose gels in TAE or TBE
- up to 400 mg of agarose can be processed per a spin column

Work in a laminar flow cabinet for DNA work.

1. Weigh a clean 1.5 ml colourless microcentrifuge tube
2. Excise the DNA fragment from the agarose gel with a clean sharp scalpel, minimize the size of the gel by removing extra agarose, place into the microcentrifuge tube
3. Re-weigh the microcentrifuge tube with the gel slice, calculate the gel weight
4. Add 3 volumes of QG buffer to 1 volume of the gel (100 mg ≈ 100 µl) (for > 2 % agarose add 6 volumes to 1 volume of the gel)
5. Incubate at 50 °C for 10 min (or until the gel slice has completely dissolved), vortex every 2 – 3 min during the incubation
6. Check if the colour of the mixture is still yellow (similar to QG buffer); in case of other colour (orange, violet), add 10 µl of 3 M sodium acetate pH 5 and mix
   (QG buffer contains a pH indicator which is yellow at pH ≤ 7.5 and orange or violet at higher pH; the following adsorption is efficient only at pH ≤ 7.5)
7. Add 1 volume of isopropanol to 1 volume of the gel
8. Apply the sample (up to 800 µl) to a QIAquick column placed in a 2 ml collection tube
9. Centrifuge at 13 000 rpm for 1 min, discard the flow;
   if the sample volume exceeds 800 µl, load the rest of the sample to the QIAquick column and spin again
10. Add 500 µl of QG buffer to the QIAquick column
11. Centrifuge at 13 000 rpm for 1 min, discard the flow
12. Add 750 µl of PE buffer to the QIAquick column, allow to stand for 2 – 5 min
13. Centrifuge at 13 000 rpm for 1 min, discard the flow and the collection tube
14. Place the QIAquick column into a new 2 ml collection tube
15. Centrifuge at 13 000 rpm for 1 min, discard the flow and the collection tube
16. Place the QIAquick column into a clean pre-labelled 1.5 ml microcentrifuge tube
17. Add 50 µl (30 – 50 µl) of EB buffer or sdH_2O directly onto the centre of QIAquick column membrane, allow to stand for 1 min
18. Centrifuge at 13 000 rpm for 1 min to elute DNA, discard the column
19. Store the DNA at - 20 °C or - 80 °C

Eventually use 1000p pipette tip, put to the gel and aspirate the band - product, put into TE buffer, leave overnight, and re-PCR (no cleaning necessary).

Protocol 24: Sequencing of PCR products or bacteria lysates
- using BigDye Terminator v3.1 Cycle Sequencing Kit Applied Biosystems

Work in a laminar flow cabinet for DNA work.
Set the reactions in singlicate, 2 reactions for 1 sample, one with the forward and one with the reverse primer.
Use sterile 0.2 ml thin-wall microcentrifuge tubes.
Thaw all the tubes (master mixes, reagents, controls, samples), gently vortex, briefly spin down, keep on ice.

DNA template	3 µl (bacterial lysate with cloned product)
	5 µl (cleaned PCR product of clonality screening)
or pGEM plasmid (200 ng / µl)	1µl (control)
Primer*) (2 pmol / µl)	1 µl (2 pmol) (cloned product)
	1.6 µl (3.2 pmol) (clonality screening)
or M13 primer (0.8 pmol / µl)	2 µl (control)
Cycle Sequencing Mix	4 µl
sdH_2O	to 20 µl

gently vortex, ev. spin down

incubation: 96°C for 60 sec
25 cycles: 96°C for 10 sec
50°C for 10 sec
60°C for 4 min
4°C at the end

(Applied Biosystems GeneAmp 2400 PCR thermal cycler, MJ PTC-200 thermal cycler)

*) when sequencing a product of a multiplex reaction (eg. PCR clonality screening) where it is not known which pair of primers produced the sequenced amplicon, a mixture of all fwd and all rvs primers (2 pmol / µl of each) may prepared and used; however, this may generate higher background;
alternatively, if there is one consensus primer (eg. JH reverse primer) perform the sequencing with this primer, determine which region (VH) is present and then perform the sequencing with the appropriate forward primer (VH forward primer); or perform the sequencing with each forward or reverse primer separately, identify the specific region/segment, and perform the sequencing with the appropriate other (reverse or forward) primer

- 3 µl of template (bacterial lysate) and 2 pmol of primers were used for sequencing of bacterial transformants to confirm the result of cloning
- 5 µl of template (cleaned PCR product) and 3.2 pmol of primers were used for sequencing of products from PCR clonality screening

Protocol 25: Purification of products of sequencing reaction
- using DyeEx 2.0 Spin Kit QIAGEN

1. Gently vortex the DyeEx 2.0 Spin column to resuspend the resin and loosen the cap of the column a quarter turn
2. Snap off the bottom closure of the Spin column and place it in a 2 ml collection tube
3. Centrifuge at 2800 rpm (750g = rcf) for 3 min, discard the flow
4. Add 300 µl of sdH_2O
5. Centrifuge at 2800 rpm (750g = rcf) for 3 min, discard the flow and the collection tube
6. Transfer the Spin column into a clean 1.5 ml microcentrifuge tube
7. Slowly apply the sequencing reaction (10 – 20 µl) directly onto the centre of the gel bed
8. Centrifuge at 2800 rpm (750g = rcf) for 3 min, discard the column
9. Dry the eluate containing purified DNA in a heated vacuum centrifuge (SpeedyVac) at medium heat for about 20 – 30 min or until the liquid has been evaporated
10. Either freeze and store at – 20 °C or proceed with running the sample on DNA sequencer

Protocol 26: Analysis on ABI 3100, examination of the sequences and ASO primers design

1. Add 10 µl of HiDi formamide to the dry pellet of the sample, resuspend with pipetting up-and-down or gentle vortexing, briefly spin down and load onto 96 well optical plate in a vertical direction; multiples of 16 wells must be filled (use HiDi formamide to fill the remaining blank wells)
2. Run on the sequencer (ABI PRISM 3100 Genetic Analyzer Applied Biosystems)
3. Analyze the sequences obtained using SeqMan II DNA Star (ABI) software, find a consensus sequence
4. Compare the consensus sequence with IG/TCR databases, identify V, D and J segments and junctional N-regions
   (IG BLAST: www.ncbi.nlm.nih.gov/igblast
   V BASE: www.mrc-cpe.cam.ac.uk/DNAPLOT.php?menu=901
   V QUEST: http://imgt.cines.fr/textes/vquest)
5. Design ASO forward primers (2 for each N-region) using Primer Express (ABI) software and manually, follow the rules:
   - length: 18 – 28 nucleotides
   - GC content: 30 – 80 %, better 40 – 60 %, similar for the primer pairs
   - melting temperature (T_m) 58 – 60 °C, with difference < 1 °C for the primer pairs, 10 °C lower than T_m of the probe
     (approximate formula – Wallace: $T_m = 2 * AT + 4 * CG$)
   - avoid runs of ≥ 4 of the same nucleotide, particularly G, if possible
   - avoid ≥ 3 G/C at the 3' end, avoid T at the 3' end
   - avoid and check for a potential of secondary structure formation (http://www.bioinfo.rpi.edu/applications/mfold/old/dna) and primer dimer formation (avoid complementarity especially at 3' ends)
   - check for homology elsewhere in the genome (http:// www.ncbi.nlm.nih.gov/BLAST/)
   - the 3' end of the primer should not include > 3 bases of the germline sequence of the J or D region, respectively

Protocol 27: DNA dilution series for primer testing and RQ-PCR quantification

Work in a laminar flow cabinet for DNA work.

1. Prepare patient's DNA and polyclonal human DNA, both at concentration 100 ng / µl
2. Pre-label 6 sterile microfuge 1.5 ml tubes, add 18 µl of human polyclonal DNA to each of them
3. Add 2 µl of the patient's DNA to tube 1, gently vortex, leave for 5 – 10 min at room temperature, exchange the pipette tip (dilution 10^{-1})
4. Take 2 µl of the contents of tube 1 and put to tube 2, gently vortex, leave for 5 – 10 min at room temperature, exchange the pipette tip (dilution 10^{-2})
5. In a similar way prepare further dilutions 10^{-3} - 10^{-6}

Protocol 28: ASO primers testing

Work in a laminar flow cabinet for DNA work.

1. Prepare 10-fold dilution series of patient's DNA in polyclonal human DNA (both at concentration 100 ng / µl), use human polyclonal DNA as a negative control
2. Set up and run RQ-PCR for the 10^{-2} dilution patient's sample, with the ASO primers and consensus Rvs primers and probes, albumin used as a control gene, all reactions in duplicates
3. Examine the Ct of the patient's sample and the control polyclonal DNA
4. For an efficient primer with supposed sensitivity of the reaction $\geq 10^{-4}$
   - the Ct of the sample (10^{-2} dilution) should be < 30
   - the difference between the Ct of the sample and Ct of the polyclonal DNA should be > 10 cycles (Ct of the polyclonal DNA must be much higher than Ct of the sample)
   - the amplification curve corresponds to a specific and efficient reaction
5. Choose the ASO primer with the best parameters (lowest Ct of the sample, biggest difference between Ct of the sample and the polyclonal control - ideally negative polyclonal control, optimal Taqman amplification curves)
6. Run the RQ-PCR with the chosen ASO primer and the whole dilution series of the patient's diagnostic sample (undiluted to 10^{-6}), human polyclonal DNA as a negative control, albumin as a control gene, all reactions in duplicates (in case of ASO primers with very good performance this could be done simultaneously with MRD detection)
7. The reproducible sensitivity of the reaction (enabling quantification) is defined as the last dilution of the patient's sample that comes up positive and where
   - its Ct is ≤ 40
   - the difference in Ct of the reaction in duplicate is ≤ 1.5 cycles
   - the mean Ct of the sample is ≥ 3 cycles lower than the mean Ct of the polyclonal DNA
   - the standard curve has a slope between 3.3 – 3.7 and correlation coefficient ≥ 0.98

   the absolute sensitivity of the reaction is defined as the last dilution of the patient's sample where
   - at least one of the reactions carried out in duplicate comes up positive
   - at least one Ct of the reactions carried out in duplicate is ≥ 1 cycle lower than the lowest Ct of the polyclonal DNA

Protocol 29: RQ-PCR for detection of Ig/TCR clonal gene rearrangements

Work in a laminar flow cabinet for DNA work.
Set the reactions in triplicate for the target gene and duplicate for the control gene and pt's serial dilution samples, for:
- all the samples
- negative control (polyclonal DNA) and no template control (sdH_2O).

Use albumin as a control gene.
Thaw all the tubes (reagents, controls, pts' samples), gently vortex, briefly spin down, keep on ice.

DNA	5 µl (500 ng; DNA 100 ng / µl)
ASO Fwd-primer (15 µM; 15 pmol/µl)	0.5 µl (300 nM)
Cons Rvs-primer (15 µM; 15 pmol/µl)	0.5 µl (300 nM)
Cons Probe (5 µM; 5 pmol/µl)	0.5 µl (100 nM)
2X TaqMan Universal Master Mix	12.5 µl
sdH_2O	to 25 µl

gently vortex, ev. spin down

incubation:	50°C for 2 min	
	95°C for 10 min	(DNA polymerase activation)
	50 cycles: 95°C for 15 sec	(denaturation)
	60°C for 60 sec	(annelation/extension)

(all indicated concentrations are for the final volume of the reaction)

data analysis: threshold set at 0.5 or 0.1
baseline set at 3 – 15 cycles

Protocol 30: Cloning of PCR products for preparation of plasmid DNA calibrators
- using TOPO TA Cloning Kit Invitrogen
(pCR 2.1-TOPO vector, TOP10 E. coli strain, chemical transformation)

Preparation of LB plates and LB medium:
Work in a sterile laminar flow cabinet for bacterial work.
LB plates:
1. Prepare and label adequate number of sterile Petri Dishes
2. Prepare LB agar (Luria agar, Miller's LB agar) by dissolving it in distilled H_2O in ç 40 g / l; 25 ml per plate
3. Sterilise LB agar by autoclaving
4. After autoclaving, pour warm LB agar (cca 25 ml per plate) in 2 Petri Dishes (control plates), then add Ampicillin 50 mg / l to the rest of LB agar, mix and pour onto the rest of plates, leave for about 30 min to solidify
5. Incubate the plates at 37 °C for 30 min
6. Spread X-Gal (50 mg / ml solution in DMF) 30 µl on each LB plate (using spreader), leave it dry for 30 min
7. Close the plates, seal around with parafilm tape, wrap in tin foil, put the plates UP SIDE DOWN, and store at 4 °C
8. Before use, incubate the plates at 37 °C for at least 30 min to be ready to use

LB medium:
1. Use LB Broth liquid medium or prepare LB Broth by dissolving Luria Broth base (Miller's modification) in distilled H_2O in ç 15.5 g / l, sterilise by autoclaving
2. Store at 4 °C

Sterilising of spreaders and bacterial wire loops
- if not using disposable spreaders and bacterial loops, sterilise in between every use:
1. Pour 100 % ethanol into one Petri dish, light the burner
2. Immerse the spreader or loop into the ethanol and take the spreader or loop twice through the flame
3. Leave to cool, check on the side of LB agar plate if it is cold

PCR reaction:
Work in a laminar flow cabinet for DNA work (start in the morning)
1. Set up the PCR reaction (using Taq polymerase, total volume 50 µl) as usual
2. Include extension step, 72 °C for 10 (7 - 30) min after the last cycle
3. Check the PCR product by electrophoresis on 2 % agarose gel, add a mass ladder to estimate the amount of the product
4. Calculate the starting amount of the PCR product:
   amount of PCR product [ng] = (size of PCR product [bp] / size of vector [bp]) x amount of vector [ng]
   here amount of vector = 10 ng, size of vector = 3931 bp, therefore
   amount of PCR product [ng] = (size of PCR product [bp] / 3931) x 10
5. Keep the PCR product on ice and continue with cloning, or freeze and store at – 20 °C (for few days)
6. Eventually consider cutting out the product from the gel and cleaning up
7. Check if the amplified gene sequence doesn't contain EcoRI target site

Cloning:
Work in a sterile laminar flow cabinet for bacterial work (start in the afternoon).
1. Use the PCR product kept on ice or thaw the stored PCR product on ice
2. Prepare and label 4 microfuge 0.5 ml tubes
   P1 (PCR product high concentration)
   P2 (PCR product medium concentration)
   P3 (PCR product low concentration)
   V1 (vector only)
3. Prepare the cloning reaction as following
   P3: PCR product - 0.5 µl or calculated starting amount
   P2: PCR product - 1 µl or 2 – 5 x starting amount
   P1: PCR product - 4 µl or 10 x starting amount
   V1: no PCR product
   to every tube: Salt solution (NaCl 1.2 M, $MgCl_2$ 0.06 M) 1 µl
   sdH_2O to the volume of 5 µl
   TOPO vector (10 mg / µl) 1 µl
   total 6 µl
4. Mix gently
5. Incubate for 5 min (30 sec – 30 min) at room temperature (22 – 23 °C)
6. Place on ice
7. Proceed with transformation of competent cells, or store at – 20 °C overnight

Transforming competent cells
Work in a sterile laminar flow cabinet for bacterial work, start in later afternoon.
1. Equilibrate a water bath to 42 °C
2. Warm the vial of SOC medium to room temperature
3. On ice thaw 6 tubes of One Shot Chemically Competent TOP10 cells and label:
   P1, P2, P3, V1, V2 (pUC19, circular vector only), E (E. coli cells only)
4. Add 2 µl of the cloning reaction into the tubes P1, P2, P3, V1; add 1 µl of pUC19 control DNA (10 pg / µl) to tube V2
5. Mix gently (by circular moving of the pipette tip or light shaking in hand), DO NOT MIX BY PIPETTING UP AN DOWN, DO NOT VORTEX!
6. Incubate on ice for 10 min (5 to 30 min)
7. Put the tubes to the water bath and incubate at 42 °C for 30 sec without shaking (heat-shock)
8. Immediately transfer the tubes onto ice (and leave for 1 – 2 min)
9. Add 250 µl of SOC medium of room temperature to each of the tube
10. Cap the tubes tightly and shake the tubes horizontally (200 rpm) at 37 °C for 1 hour
11. From tubes P1 - 3 prepare 1:10 dilution in SOC (90 µl of SOC + 10 µl of the cell suspension; in sterile microfuge tubes)
12. Spread 50 µl from tubes P1 – 3, their dilutions, and V1 - 2 on pre-warmed LB plates with Ampicillin using a sterile spreader; spread 50 µl of tube E on 2 LB plates, one with and one without Ampicillin
13. Seal the plates around with parafilm, cover with tin foil, put up side down and incubate at 37 °C for about 12 hours

Testing transformants by PCR

Work in a sterile laminar flow cabinet for bacterial work (start in morning).

1. Prepare complete PCR master mix for the cloned gene (the same reaction used to generate the PCR product for the cloning)
2. Aliquot the PCR master mix into pre-labelled 10 sterile microfuge 0.5 ml tubes (48 µl per tube)
3. Pre-warm one LB plate with Ampicillin with a grid on its back
4. Pick up to 10 white or light blue colonies from the plates P1 – P3 or their dilution for further analysis
5. With a pipette tip, transfer them onto the new LB plate with a grid (several dots or streaks with the pipette tip per 1 colony transferred onto 1 square of the plate)
6. Put the pipette tip into a tube with PCR reaction and mix well by pipetting up and down
7. Seal the plate around with parafilm, wrap in tin foil, put up side down, incubate at 37 °C for about 12 – 24 hours, then store at 4 °C
8. Perform the PCR reaction and analyse the products by electrophoresis on 2 % agarose gel to identify the colonies containing the cloned sequence

Testing transformants by sequencing

Work in a sterile laminar flow cabinet for bacterial work (start in morning).

1. Prepare 2 - 3 sterile microfuge 0.5 tubes with 20 µl of sdH_2O
2. Pre-warm LB medium for 37°C
3. Prepare and label 2 – 3 Sarstedt 50 ml tubes
4. Add 5 ml of LB medium and Ampicillin 50 µg / ml to each Sarstedt 50 ml tube
5. From the plate with grid pick up 2 – 3 original colonies that gave the best results on PCR (for each original colony there should be now several daughter colonies within the one square)
6. For each of the 2 – 3 original colonies, put (by pipette tip) 1 daughter colony to the microfuge 0.5 ml tube and 1 colony into the Sarstedt 50 ml tube
7. Seal the plate around with parafilm, wrap in tin foil, put up side down and keep at 4 °C
8. Incubate the Sarstedt tubes at 37°C on a shaker for about 8 - 12 hours (starting culture)
9. Incubate the microfuge tubes at 98°C for 20 min (in PCR machine) to lyse the bacteria, vortex and briefly spin down
10. Use 3 µl of this bacteria lysate as a template for sequencing reaction
11. Perform the sequencing reaction according to the standard protocol, using M13 Fwd and M13 Rvs primers (2 pmol of each) included in the TOPO TA Cloning kit, and using pGEM as a positive control

If the sequencing gives good results, go to the next step, if not, pick up other colonies from the LB plate and repeat sequencing with these

Preparing a stock of transformants and growing transformants for plasmid DNA production:

Work in a sterile laminar flow cabinet for bacterial work (start in evening).

Preparing a stock of transformants

1. Use the Sarstedt 50 ml tubes containing 5 ml of the starting culture
2. Prepare and label 2 sterile Sarstedt 2 ml tubes per 1 clone (per Sarstedt 50 ml tube), work on ice
3. Add 150 µl of sterile glycerol to each tube
4. Add 850 µl of the culture to the tube, mix gently by vortexing
5. Store at - 80°C

Growing transformants for plasmid DNA production

1. Use the rest of the starting culture in Sarstedt 50 ml tubes
2. Pre-warm LB medium and prepare 250 ml sterile plastic flask(s) for bacterial cultures
3. Put 50 ml of LB medium into the flask(s), add Ampicillin 50 µg / ml
4. Put 100 µl of the starting culture to the flask(s)
5. Grow at 37 °C on a shaker (300 rpm) for 12 – 16 hours (overnight)

Protocol 31: Extraction and purification of plasmid DNA
- using HiSpeed Plasmid Midi Kit QIAGEN

Before starting:
- add RNase A solution to Buffer P1
- check Buffer P2 for SDS precipitation and, if necessary, dissolve by warming to 37°C
- pre-chill Buffer P3 to 4°C

1. Put the bacterial culture from the flask(s) to sterile tube(s)
2. Centrifuge at 6 000 g for 15 min at 4 °C, discard the supernatant
3. Freeze down the cell pellet at – 20 °C or proceed with DNA extraction
4. Add 6 ml of Buffer P1 to the bacterial pellet and resuspend it thoroughly by vortexing or pipetting up and down (so no cell clumps remain)
5. Add 6 ml of Buffer P2, mix gently but thoroughly by inverting 4 – 6 times (to get viscous lysate), DO NOT VORTEX, CLOSE THE BUFFER P2 BOTTLE IMMEDIATELY AFTER USE
6. Incubate at room temperature for 5 min
7. Prepare QIAfilter cartridges: screw the cap onto the outlet nozzle of the cartridge, place the cartridge into a Sarstedt 50 ml tube
8. Add 6 ml of chilled Buffer P3, mix immediately and gently by inverting 4 – 6 times (a fluffy white precipitate should occur, otherwise more mixing is required)
9. Immediately pour the lysate into the barrel of the QIAfilter cartridge, do not insert the plunger
10. Incubate at room temperature for 10 min (a precipitate should form a layer on top of the solution; if not, carefully run a sterile pipette tip around the walls of the cartridge to dislodge it)
11. Equilibrate a HiSpeed Tip by applying 4 ml of Buffer QBT and allow the column to empty by gravity flow, discard the flow, put the tip into a new 50 ml tube
12. Remove the cap from the QIAfilter outlet nozzle, gently insert the plunger into the cartridge and filter all the cell lysate into the previously equilibrated HiSpeed Tips (about 15 ml of cleared lysate should be recovered)
13. Allow the lysate to enter the resin by gravity flow, discard the flow
14. Apply 20 ml of Buffer QC into the HiSpeed Tip, allow it to move through by gravity flow, discard the flow (washing)
15. Put the HiSpeed Tip into a sterile tube (20 – 50 ml)
16. Apply 5 ml of Buffer QF to elute DNA, allow it to move through by gravity flow
17. Add 3.5 ml of room-temperature isopropanol to the eluate to precipitate DNA, mix
18. Incubate for 5 min at room temperature
19. Remove the plunger from a 20 ml syringe and, after that, gently attach the QIAprecipitator Module onto the outlet nozzle
20. Place the QIAprecipitator over a waste bottle
21. Transfer the eluate/isopropanol mixture into the syringe, insert the plunger and filter the mixture through the QIAprecipitator using constant pressure
22. Remove the QIAprecipitator from the syringe and pull out the plunger
23. Re-attach the QIA precipitator and add 2 ml of 70 % ethanol to the syringe, insert the plunger and press the ethanol through the QIAprecipitator using constant pressure (to wash the DNA)
24. Remove the QIAprecipitator from the syringe, pull out the plunger
25. Attach the QIAprecipitator back to the syringe, insert the plunger and press air through the QIAprecipitator quickly and forcefully (to dry the membrane)
26. Repeat steps 24. and 25.
27. Dry the outlet nozzle of the QIAprecipitator with tissue to prevent ethanol carry-over
28. Remove the plunger from a new 5 ml syringe and gently attach the QIAprecipitator onto the outlet nozzle
29. Hold the outlet of the QIAprecipitator over a sterile microfuge 1.5 ml tube, add 1 ml (or 0.5 ml if higher concentration of DNA is required) of Buffer TE to the syringe, insert the plunger and elute the DNA into the collection tube using constant pressure
30. Remove the QIAprecipitator from the syringe, pull out the plunger, reattach the QIAprecipitator to the syringe
31. Transfer the previous eluate to the syringe and elute for a second time into the same microfuge 1.5 ml tube
32. Determine the yield by SPEC (up to 0.2 µg / µl may be expected)
33. Run the extracted DNA on 1 % agarose gel with appropriate ladder to check for purity (supercoiled form or bacterial genomic DNA contamination may give additional bands of higher size; supercoiled form will disappear after restriction)
34. Keep and store part of the amount of the circular DNA plasmid at – 20 °C or – 80 °C, use the rest for other applications (linearising)

Protocol 32: Linearising of plasmid DNA and preparing a stock of calibrators

Linearising of plasmid DNA by restriction enzyme digestion

1. Quantify the concentration of extracted plasmid DNA by SPEC (reading should be between 0.1 and 1.0, if not dilute or concentrate to get a precise quantification)
2. Calculate the copy number per 1 µg of DNA

$$\text{copy number [molecules / µl]} = \frac{\text{c DNA [µg / µl]}}{\text{n [bp]}} \times \frac{6.022 \times 10^{17}}{660}$$

where n = size of DNA: size of the vector + size of the insert

3. Set up the reaction:

DNA	20 µg	(1 or 2 or 5 µg)
REact3	40 µl	(2 µl)
EcoRI (10 U/µl)	10 µl	(0.5 or 1 or 2.5 µl)
sdH_2O	to 400 µl	(to 20 µl)

4. Incubate in PCR machine for 1 hour at 37 °C
5. Run on 1 % agarose gel (with undigested plasmid as a control) to check the efficiency of the digestion (5 µl of both undigested and linearised product)

Preparing a stock of calibrators

1. Prepare a diluting solution:

Tris	10 mM
EDTA	1 mM
E.coli rRNA in sdH_2O ($pH \approx 8$)	20 µg / ml

2. Prepare 6 serial dilutions of the linearised plasmids in the diluting solution containing 2, 20, 200, 2 000, 20 000 and 200 000 copies of the gene / 1 µl
3. Store at – 20 °C

Protocol 33: FC combined indirect and direct staining for intracellular antigens (using IntraStain DAKO kit)

1. Dilute whole bone marrow or peripheral blood sample, separated mononuclears or cell-line cells in CellWash/FBS to the concentration of 1 x 10^7 cells / ml
2. Prepare and label Falcon 5 ml polystyrene tubes
3. Add 100 µl of the sample to each tube, vortex
4. Add 100 µl of IntraStain (DAKO) Reagent A – Fixation , vortex
5. Incubate at room temperature in the dark for 15 min
6. Add 2 ml of CellWash/FBS, vortex
7. Centrifuge at 400 g for 5 min, discard the supernatant, vortex the pellet
8. Add primary unlabelled monoclonal antibody (mouse anti-human) against the intracellular antigen
9. Add 100 µl of IntraStain (DAKO) Reagent B – Permeabilisation , vortex
10. Incubate at room temperature in the dark for 15 min
11. Add 2 ml of CellWash/FBS, vortex
12. Centrifuge at 400 g for 5 min, discard the supernatant
13. Add secondary fluorochrome-labelled monoclonal antibody (goat anti-mouse) against the primary antibody
14. Add 100 µl of IntraStain (DAKO) Reagent B – Permeabilisation , vortex
15. Incubate at room temperature in the dark for 15 min
16. Add 2 ml of CellWash/FBS, vortex
17. Centrifuge at 400 g for 5 min, discard the supernatant
18. Add normal mouse serum in an amount equal to that of the secondary antibody and 100 µl of CellWash/FBS, vortex
19. Incubate at room temperature in the dark for 15 min
20. Add 2 ml of CellWash/FBS, vortex
21. Centrifuge at 400 g for 5 min, discard the supernatant, vortex the pellet
22. Add directly labelled monoclonal antibody(ies) against surface or intracellular antigen, vortex
23. In case of staining for intracellular antigen(s) add 100 µl of IntraStain (DAKO) Reagent B – Permeabilisation , vortex
24. Incubate at room temperature in the dark for 12 - 15 min
25. Add 2 ml of CellWash/FBS, vortex
26. Centrifuge at 400 g for 5 min, discard the supernatant
27. Add 0.5 ml of CellFix (diluted 1:10 in sdH_2O), vortex
28. Keep in the dark
29. Acquire immediately or keep at 4°C in the dark until acquisition, acquire within 4 hours

If doing indirect staining for an intracellular antigen only, skip steps 18. – 24.

Protocol 34: FC staining for WT1 protein
- using Fix and Perm Caltag kit

1. Dilute whole bone marrow or peripheral blood sample or separated mononuclears in CellWash/FBS to the WBC concentration of 1 x 10^7 / ml
2. Prepare and label Falcon 5 ml polystyrene tubes
3. Add monoclonal antibodies against surface markers to the tubes (if required)
4. Add 100 µl of the sample to each tube, vortex
5. Incubate at room temperature in the dark for 12 min
6. Add 2 ml of CellWash, vortex
7. Centrifuge at 400 g for 5 min, discard the supernatant, vortex the pellet
8. Add 100 µl of Fix and Perm (Caltag) Reagent A – Fixation , vortex
9. Incubate at room temperature in the dark for 15 min
10. Add 2 ml of CellWash, vortex
11. Centrifuge at 400 g for 5 min, discard the supernatant, vortex the pellet
12. Add WT1-AlexaFluor488 and isotype control (eventually other antibodies against intracellular markers) to the tubes
13. Add 100 µl of Fix and Perm (Caltag) Reagent B – Permeabilisation , vortex
14. Incubate at room temperature in the dark for 20 min
15. Add 2 ml of CellWash, vortex
16. Centrifuge at 400 g for 5 min, discard the supernatant
17. Add 0.5 ml of CellFix (diluted 1:10 in sdH_2O), vortex
18. Keep in the dark
19. Acquire immediately or keep at 4°C in the dark until acquisition, acquire within 8 hours

Protocol 35: Immunofluorescent staining for WT1 protein

1. Prepare a suspension of cells (cell line, separated mononuclears) in PBS/BSA at concentration 1 x 10^6 / ml
2. Take 100 µl of the cell suspension and centrifuge onto a glass slide using cytospin, at 700 rpm for 2 min
3. Incubate the slides in ice-cold 100 % methanol, at 4 °C for 10 min
4. Wash in PBS/BSA, 3 x 5 min
5. Incubate the slides in PBS/BSA/Triton X-100 0.5 % for 20 min
6. Wash in PBS/BSA, 3 x 5 min
7. Add fluorochrome-labelled anti-WT1 antibody diluted in PBS/BSA/SA (Ig concentration 10 µg / ml) onto the slides and incubate in a humidified chamber, at 37 °C in the dark for 1 hour
8. Wash in PBS/BSA, 3 x 5 min
9. Apply fluorescent mounting medium (DAKO) and a cover glass, keep at 4 °C in the dark
10. Examine under a fluorescent microscope

Reagents used and their sources:

General reagents:

Ethanol *(Sigma-Aldrich)*
Methanol *(Sigma-Aldrich)*
Glycerol *(Sigma-Aldrich)*
Isopropanol *(Sigma-Aldrich)*
Tris/EDTA *(Sigma-Aldrich)*
D-PBS *(Invitrogen/Gibco)*
RPMI 1640 *(Invitrogen/Gibco)*
RPMI 1640 with Glutamax *(Invitrogen/Gibco)*
HBSS *(Invitrogen/Gibco)*
Foetal Bovine Serum *(Invitrogen/Gibco)*
Dimethyl Sulfoxide *(Sigma-Aldrich)*
Bovine Serum Albumin *(Sigma-Aldrich)*
Sodium Azide *(Sigma-Aldrich)*
Triton-X *(Sigma-Aldrich)*
Lymphoprep *(Axis-Shield)*
Ethidium Bromide *(Sigma-Aldrich)*
Acridine Orange *(Sigma-Aldrich)*
Penicillin/Streptomycin *(Invitrogen/Gibco)*
Luria Agar *(Sigma-Aldrich)*
Luria Broth (Miler's Modification) *(Sigma-Aldrich)*
X-Gal *(Promega)*

Flow cytometry reagents:

FACSFlow *(Becton Dickinson)*
CellWash *(Becton Dickinson)*
CellFix *(Becton Dickinson)*
FACS Lysing solution *(Becton Dickinson)*
Fix and Perm *(Caltag)*
Intrastain *(Dako)*
AlexaFluor 488 *(Molecular Probes)*
antibodies - see Table 4.5.

PCR and post-PCR reagents:

primers *(MWG-Biotech)*
TaqMan probes *(Applied Biosystems)*
SuperScript II RNase H Reverse Transcriptase *(Invitrogen)*
AmpliTaq Gold *(Applied Biosystems)*
TaqMan Universal PCR MasterMix *(Applied Biosystems)*
Pre-Developed TaqMan Assay Reagents Control Kits B2M *(Applied Biosystems)*
FusionQuant Standards *(Ipsogen)*
Uracil-DNA Glycosylase, heat-labile *(Roche)*
Recombinant RNasin Ribonuclease Inhibitor *(Promega)*
dUTP *(Promega)*
dNTPs *(Promega)*
100 bp DNA Ladder *(Promega)*
Low DNA Mass Ladder *(Invitrogen)*
IGH Gene Clonality Assay - Gel Detection *(InVivo Scribe)*
IGH Gene Clonality Assay - ABI Fluorescence Detection *(InVivo Scribe)*
IGK Gene Clonality Assay - Gel Detection *(InVivo Scribe)*
IGK Gene Clonality Assay - ABI Fluorescence Detection *(InVivo Scribe)*
TCRG Gene Clonality Assay - Gel Detection *(InVivo Scribe)*
TCRG Gene Clonality Assay - ABI Fluorescence Detection *(InVivo Scribe)*
TCRD Gene Clonality Assay - Gel Detection *(InVivo Scribe)*
TCRD Gene Clonality Assay - ABI Fluorescence Detection *(InVivo Scribe)*
ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit *(Applied Biosystems)*
Hi-Di Formamide *(Applied Biosystems)*
GeneScan 400HD ROX Size Standard *(Applied Biosystems)*
EcoR I *(Invitrogen)*
Agarose *(Invitrogen)*
MetaPhore Agarose *(Cambrex)*
Tris Acetate EDTA *(Sigma-Aldrich)*
Tris Borate EDTA *(Sigma-Aldrich)*

9. References

Advani, A. S. and A. M. Pendergast (2002). "Bcr-Abl variants: biological and clinical aspects." Leuk Res **26**(8): 713-20.

Afonja, O., J. E. Smith, Jr., et al. (2000). "MEIS1 and HOXA7 genes in human acute myeloid leukemia." Leuk Res **24**(10): 849-55.

Alcalay, M., A. Orleth, et al. (2001). "Common themes in the pathogenesis of acute myeloid leukemia." Oncogene **20**(40): 5680-94.

Allen, T. D., Y. X. Zhu, et al. (2000). "TALE homeoproteins as HOX11-interacting partners in T-cell leukemia." Leuk Lymphoma **39**(3-4): 241-56.

Allman, D., J. C. Aster, et al. (2002). "Notch signaling in hematopoiesis and early lymphocyte development." Immunol Rev **187**: 75-86.

Anastasiadou, E. and J. Schwaller (2003). "Role of constitutively activated protein tyrosine kinases in malignant myeloproliferative disorders: an update." Curr Opin Hematol **10**(1): 40-8.

Arlinghaus, R. B. (2002). "Bcr: a negative regulator of the Bcr-Abl oncoprotein in leukemia." Oncogene **21**(56): 8560-7.

Armstrong, S. A., J. E. Staunton, et al. (2002). "MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia." Nat Genet **30**(1): 41-7.

Armstrong, S. A., A. L. Kung, et al. (2003). "Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification." Cancer Cell **3**(2): 173-83.

Arrigoni, P., C. Beretta, et al. (2003). "FLT3 internal tandem duplication in childhood acute myeloid leukaemia: association with hyperleucocytosis in acute promyelocytic leukaemia." Br J Haematol **120**(1): 89-92.

Aster, J. C. (2005). "Deregulated NOTCH signaling in acute T-cell lymphoblastic leukemia/lymphoma: new insights, questions, and opportunities." Int J Hematol. **82**(4): 295-301.

Azuma, T., M. Makita, et al. (2002). "Identification of a novel WT1-derived peptide which induces human leucocyte antigen-A24-restricted anti-leukaemia cytotoxic T lymphocytes." Br J Haematol **116**(3): 601-3.

Bader, P., J. Hancock, et al. (2002). "Minimal residual disease (MRD) status prior to allogeneic stem cell transplantation is a powerful predictor for post-transplant outcome in children with ALL." Leukemia **16**(9): 1668-72.

Baer, M. R., C. C. Stewart, et al. (2001). "High frequency of immunophenotype changes in acute myeloid leukemia at relapse: implications for residual disease detection (Cancer and Leukemia Group B Study 8361)." Blood **97**(11): 3574-80.

Bain, B. J., D. Barnett, et al. (2002). "Revised guideline on immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders." Clin Lab Haematol **24**(1): 1-13.

Baker, K. S., T. E. DeFor, et al. (2003). "New malignancies after blood or marrow stem-cell transplantation in children and adults: incidence and risk factors." J Clin Oncol **21**(7): 1352-8.

Ballerini, P., A. Blaise, et al. (2002). "HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis." Blood **100**(3): 991-7.

Ballerini, P., A. Blaise, et al. (2003). "A novel real-time RT-PCR assay for quantification of OTT-MAL fusion transcript reliable for diagnosis of t(1;22) and minimal residual disease (MRD) detection." Leukemia **17**(6): 1193-6.

Barragan, E., J. Cervera, et al. (2004). "Prognostic implications of Wilms' tumor gene (WT1) expression in patients with de novo acute myeloid leukemia." Haematologica **89**(8): 926-933.

Beillard, E., N. Pallisgaard, et al. (2003). "Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase

polymerase chain reaction (RQ-PCR) - a Europe against cancer program." Leukemia **17**(12): 2474-86.

Bellantuono, I., L. Gao, et al. (2002). "Two distinct HLA-A0201-presented epitopes of the Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL." Blood **100**(10): 3835-7.

Bene, M. C., M. Bernier, et al. (1999). "Impact of immunophenotyping on management of acute leukemias." Haematologica **84**(11): 1024-34.

Bene, M. C., M. Bernier, et al. (2001). "Acute myeloid leukaemia M0: haematological, immunophenotypic and cytogenetic characteristics and their prognostic significance: an analysis in 241 patients." Br J Haematol **113**(3): 737-45.

Bergmann, L., C. Miething, et al. (1997). "High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome." Blood **90**(3): 1217-25.

Berman, E. (2000). "Recent advances in the treatment of acute leukemia: 1999." Curr Opin Hematol **7**(4): 205-11.

Bhatia, S., H. N. Sather, et al. (2002). "Low incidence of second neoplasms among children diagnosed with acute lymphoblastic leukemia after 1983." Blood **99**(12): 4257-64.

Biondi, A., G. Cimino, et al. (2000a). "Biological and therapeutic aspects of infant leukemia." Blood **96**(1): 24-33.

Biondi, A., M. G. Valsecchi, et al. (2000b). "Molecular detection of minimal residual disease is a strong predictive factor of relapse in childhood B-lineage acute lymphoblastic leukemia with medium risk features. A case control study of the International BFM study group." Leukemia **14**(11): 1939-43.

Bjorklund, E., J. Mazur, et al. (2003). "Flow cytometric follow-up of minimal residual disease in bone marrow gives prognostic information in children with acute lymphoblastic leukemia." Leukemia **17**(1): 138-48.

Blobel, G. A. (2000). "CREB-binding protein and p300: molecular integrators of hematopoietic transcription." Blood **95**(3): 745-55.

Boeckx, N., M. J. Willemse, et al. (2002). "Fusion gene transcripts and Ig/TCR gene rearrangements are complementary but infrequent targets for PCR-based detection of minimal residual disease in acute myeloid leukemia." Leukemia **16**(3): 368-75.

Boissel, N., J. M. Cayuela, et al. (2002). "Prognostic significance of FLT3 internal tandem repeat in patients with de novo acute myeloid leukemia treated with reinforced courses of chemotherapy." Leukemia **16**(9): 1699-704.

Borowitz, M. J., D. J. Pullen, et al. (2003). "Minimal residual disease detection in childhood precursor-B-cell acute lymphoblastic leukemia: relation to other risk factors. A Children's Oncology Group study." Leukemia **17**(8): 1566-72.

Bowen, D. T., M. E. Frew, et al. (2003). "CYP1A1*2B (Val) allele is overrepresented in a subgroup of acute myeloid leukemia patients with poor-risk karyotype associated with NRAS mutation, but not associated with FLT3 internal tandem duplication." Blood **101**(7): 2770-4.

Boxer, L. M. and C. V. Dang (2001). "Translocations involving c-myc and c-myc function." Oncogene **20**(40): 5595-610.

Bruening, W. and J. Pelletier (1996). "A non-AUG translational initiation event generates novel WT1 isoforms." J Biol Chem. **271**(15): 8646-54.

Bruggemann, M., J. Droese, et al. (2000). "Improved assessment of minimal residual disease in B cell malignancies using fluorogenic consensus probes for real-time quantitative PCR." Leukemia **14**(8): 1419-25.

Bruggemann, M., V. H. van der Velden, et al. (2004). "Rearranged T-cell receptor beta genes represent powerful targets for quantification of minimal residual disease in childhood and adult T-cell acute lymphoblastic leukemia." Leukemia **18**(4): 709-19.

Burnett, A. K. (1999). "Tailoring the treatment of acute myeloid leukemia." Curr Opin Hematol **6**(4): 247-52.

Buske, C. and R. K. Humphries (2000). "Homeobox genes in leukemogenesis." Int J Hematol **71**(4): 301-8.

Campana, D. and C. H. Pui (1995). "Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance." Blood **85**(6): 1416-34.

Campana, D. and E. Coustan-Smith (1999). "Detection of minimal residual disease in acute leukemia by flow cytometry." Cytometry **38**(4): 139-52.

Campana, D. and F. G. Behm (2000). "Immunophenotyping of leukemia." J Immunol Methods **243**(1-2): 59-75.

Campana, D., G. A. Neale, et al. (2001). "Detection of minimal residual disease in acute lymphoblastic leukemia: the St Jude experience." Leukemia **15**(2): 278-9.

Campana, D. and E. Coustan-Smith (2002). "Advances in the immunological monitoring of childhood acute lymphoblastic leukaemia." Best Pract Res Clin Haematol **15**(1): 1-19.

Campana, D. (2003). "Determination of minimal residual disease in leukaemia patients." Br J Haematol **121**(6): 823-38.

Campana, D., E. Coustan-Smith, et al. (2004). "Minimal residual disease studies by flow cytometry in acute leukemia." Acta Haematol **112**(1-2): 8-15.

Campbell, C. E., A. Huang, et al. (1994). "Antisense transcripts and protein binding motifs within the Wilms tumour (WT1) locus." Oncogene. **9**(2): 583-95.

Cantor, A. B. (2005). "GATA transcription factors in hematologic disease." Int J Hematol. **81**(5): 378-84.

Caricasole, A., A. Duarte, et al. (1996). "RNA binding by the Wilms tumor suppressor zinc finger proteins." Proc Natl Acad Sci U S A **93**(15): 7562-6.

Carlesso, N., J. C. Aster, et al. (1999). "Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics." Blood **93**(3): 838-48.

Carpenter, B., K. J. Hill, et al. (2004). "BASP1 is a transcriptional cosuppressor for the Wilms' tumor suppressor protein WT1." Mol Cell Biol **24**(2): 537-49.

Carter, T. L., P. M. Watt, et al. (2001). "Hemizygous p16(INK4A) deletion in pediatric acute lymphoblastic leukemia predicts independent risk of relapse." Blood **97**(2): 572-4.

Cave, H., J. van der Werff ten Bosch, et al. (1998). "Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer--Childhood Leukemia Cooperative Group." N Engl J Med **339**(9): 591-8.

Cazzaniga, G., E. d'Aniello, et al. (2002a). "Results of minimal residual disease (MRD) evaluation and MRD-based treatment stratification in childhood ALL." Best Pract Res Clin Haematol **15**(4): 623-38.

Cazzaniga, G., V. Rossi, et al. (2002b). "Monitoring minimal residual disease using chromosomal translocations in childhood ALL." Best Pract Res Clin Haematol **15**(1): 21-35.

Cazzaniga, G. and A. Biondi (2005). "Molecular monitoring of childhood acute lymphoblastic leukemia using antigen receptor gene rearrangements and quantitative polymerase chain reaction technology." Haematologica. **90**(3): 382-90.

Chang, F., L. S. Steelman, et al. (2003). "Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention." Leukemia **17**(7): 1263-93.

Chase, A., A. Reiter, et al. (1999). "Fusion of ETV6 to the caudal-related homeobox gene CDX2 in acute myeloid leukemia with the t(12;13)(p13;q12)." Blood **93**(3): 1025-31.

Chen, J. S., E. Coustan-Smith, et al. (2001a). "Identification of novel markers for monitoring minimal residual disease in acute lymphoblastic leukemia." Blood **97**(7): 2115-20.

Chen, Z. (2001b). "The possible role and application of WT1 in human leukemia." Int J Hematol **73**(1): 39-46.

Chiba, S. (1998). "Homeobox genes in normal hematopoiesis and leukemogenesis." Int J Hematol **68**(4): 343-53.

Christiansen, D. H. and J. Pedersen-Bjergaard (2001). "Internal tandem duplications of the FLT3 and MLL genes are mainly observed in atypical cases of therapy-related acute myeloid leukemia with a normal karyotype and are unrelated to type of previous therapy." Leukemia **15**(12): 1848-51.

Cilloni, D., E. Gottardi, et al. (2002). "Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients." Leukemia **16**(10): 2115-21.

Cilloni, D., E. Gottardi, et al. (2003). " Usefulness of quantitative assessment of the WT1 gene transcript as a marker for minimal residual disease detection." Blood **102**(2): 773-4.

Cilloni, D. and G. Saglio (2004). "WT1 as a universal marker for minimal residual disease detection and quantification in myeloid leukemias and in myelodysplastic syndrome." Acta Haematol **112**(1-2): 79-84.

Cimino, G., L. Elia, et al. (2000). "A prospective study of residual-disease monitoring of the ALL1/AF4 transcript in patients with t(4;11) acute lymphoblastic leukemia." Blood **95**(1): 96-101.

Ciudad, J., J. F. San Miguel, et al. (1998). "Prognostic value of immunophenotypic detection of minimal residual disease in acute lymphoblastic leukemia." J Clin Oncol **16**(12): 3774-81.

Clark, J. J., F. O. Smith, et al. (2003). "Update in childhood acute myeloid leukemia: recent developments in the molecular basis of disease and novel therapies." Curr Opin Hematol **10**(1): 31-9.

Codrington, R., H. E. O'Connor, et al. (2000). "Analysis of ETV6/AML1 abnormalities in acute lymphoblastic leukaemia: incidence, alternative spliced forms and minimal residual disease value." Br J Haematol **111**(4): 1071-9.

Countouriotis, A., T. B. Moore, et al. (2002). "Cell surface antigen and molecular targeting in the treatment of hematologic malignancies." Stem Cells **20**(3): 215-29.

Coustan-Smith, E., J. Sancho, et al. (2000). "Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia." Blood **96**(8): 2691-6.

Coustan-Smith, E., J. Sancho, et al. (2002a). "Use of peripheral blood instead of bone marrow to monitor residual disease in children with acute lymphoblastic leukemia." Blood **100**(7): 2399-402.

Coustan-Smith, E., J. Sancho, et al. (2002b). "Prognostic importance of measuring early clearance of leukemic cells by flow cytometry in childhood acute lymphoblastic leukemia." Blood **100**(1): 52-8.

Coustan-Smith, E., R. C. Ribeiro, et al. (2003). "Clinical significance of residual disease during treatment in childhood acute myeloid leukaemia." Br J Haematol **123**(2): 243-52.

Coustan-Smith, E., A. Gajjar, et al. (2004). "Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia after first relapse." Leukemia **18**(3): 499-504.

Crans-Vargas, H. N., E. M. Landaw, et al. (2002). "Expression of cyclic adenosine monophosphate response-element binding protein in acute leukemia." Blood **99**(7): 2617-9.

Crispino, J. D. (2005). "GATA1 in normal and malignant hematopoiesis." Semin Cell Dev Biol. **16**(1): 137-47. Epub 2004 Dec 13.

Dalle, J. H., M. Fournier, et al. (2002). "p16(INK4a) immunocytochemical analysis is an independent prognostic factor in childhood acute lymphoblastic leukemia." Blood **99**(7): 2620-3.

Dallosso, A. R., A. L. Hancock, et al. (2004). "Genomic imprinting at the WT1 gene involves a novel coding transcript (AWT1) that shows deregulation in Wilms' tumours." Hum Mol Genet. **13**(4): 405-15. Epub 2003 Dec 17.

Dame, C., K. M. Kirschner, et al. (2006). "Wilms tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene." Blood. **107**(11): 4282-90. Epub 2006 Feb 7.

Dash, A. and D. G. Gilliland (2001). "Molecular genetics of acute myeloid leukaemia." Best Pract Res Clin Haematol **14**(1): 49-64.

de Haas, V., O. J. Verhagen, et al. (2001). "Quantification of minimal residual disease in children with oligoclonal B-precursor acute lymphoblastic leukemia indicates that the clones that grow out

during relapse already have the slowest rate of reduction during induction therapy." Leukemia **15**(1): 134-40.

de Haas, V., R. J. Vet, et al. (2002). "Early detection of central nervous system relapse by polymerase chain reaction in children with B-precursor acute lymphoblastic leukemia." Ann Hematol **81**(1): 59-61.

Dechsukhum, C., J. L. Ware, et al. (2000). "Detection of a novel truncated WT1 transcript in human neoplasia." Mol Diagn **5**(2): 117-28.

Degos, L., D. C. Linch, et al. (1999). Textbook of malignant haematology. London, Martin Dunitz.

Deneys, V., A. M. Mazzon, et al. (2001). "Reference values for peripheral blood B-lymphocyte subpopulations: a basis for multiparametric immunophenotyping of abnormal lymphocytes." J Immunol Methods **253**(1-2): 23-36.

Devlin, T. (1997). Textbook of biochemistry : with clinical correlations. New York, Wiley-Liss.

Dhut, S., E. L. Dorey, et al. (1988). "Identification of two normal bcr gene products in the cytoplasm." Oncogene **3**(5): 561-6.

Diverio, D., V. Rossi, et al. (1998). "Early detection of relapse by prospective reverse transcriptase-polymerase chain reaction analysis of the PML/RARalpha fusion gene in patients with acute promyelocytic leukemia enrolled in the GIMEMA-AIEOP multicenter "AIDA" trial. GIMEMA-AIEOP Multicenter "AIDA" Trial." Blood **92**(3): 784-9.

Donadieu, J. and C. Hill (2001). "Early response to chemotherapy as a prognostic factor in childhood acute lymphoblastic leukaemia: a methodological review." Br J Haematol **115**(1): 34-45.

Downing, J. R. (2001). "AML1/CBFbeta transcription complex: its role in normal hematopoiesis and leukemia." Leukemia **15**(4): 664-5.

Drabkin, H. A., C. Parsy, et al. (2002). "Quantitative HOX expression in chromosomally defined subsets of acute myelogenous leukemia." Leukemia **16**(2): 186-95.

Drummond, I. A., H. D. Rupprecht, et al. (1994). "DNA recognition by splicing variants of the Wilms' tumor suppressor, WT1." Mol Cell Biol. **14**(6): 3800-9.

Dumur, C. I., C. Dechsukhum, et al. (2002). "Analytical validation of a real-time reverse transcription-polymerase chain reaction quantitation of different transcripts of the Wilms' tumor suppressor gene (WT1)." Anal Biochem **309**(1): 127-36.

Dworzak, M. N., G. Fritsch, et al. (1997). "Multiparameter phenotype mapping of normal and post-chemotherapy B lymphopoiesis in pediatric bone marrow." Leukemia **11**(8): 1266-73.

Dworzak, M. N., G. Froschl, et al. (2002). "Prognostic significance and modalities of flow cytometric minimal residual disease detection in childhood acute lymphoblastic leukemia." Blood **99**(6): 1952-8.

Dworzak, M. N. and E. R. Panzer-Grumayer (2003). "Flow cytometric detection of minimal residual disease in acute lymphoblastic leukemia." Leuk Lymphoma. **44**(9): 1445-55.

Eckert, C., A. Biondi, et al. (2001). "Prognostic value of minimal residual disease in relapsed childhood acute lymphoblastic leukaemia." Lancet **358**(9289): 1239-41.

Eichler-Reiss, F., P. Reizenstein, et al. (1985). "Influence of pre-incubation with one antibody on the binding of a second by human lymphocytes." Med Oncol Tumor Pharmacother **2**(4): 287-90.

Einsiedel, H. G., T. Taube, et al. (2001). "Absence of mutations in the CDKN2 binding site of CDK4 in childhood acute lymphoblastic leukemia." Leuk Lymphoma **40**(3-4): 413-7.

Elia, L., M. Mancini, et al. (2003). "A multiplex reverse transcriptase-polymerase chain reaction strategy for the diagnostic molecular screening of chimeric genes: a clinical evaluation on 170 patients with acute lymphoblastic leukemia." Haematologica **88**(3): 275-9.

Elisseeva, O. A., Y. Oka, et al. (2002). "Humoral immune responses against Wilms tumor gene WT1 product in patients with hematopoietic malignancies." Blood **99**(9): 3272-9.

Ellisen, L. W., N. Carlesso, et al. (2001). "The Wilms tumor suppressor WT1 directs stage-specific quiescence and differentiation of human hematopoietic progenitor cells." Embo J **20**(8): 1897-909.

Elmaagacli, A. H., M. Koldehoff, et al. (2005). "WT1 and BCR-ABL specific small interfering RNA have additive effects in the induction of apoptosis in leukemic cells." Haematologica. **90**(3): 326-34.

Englert, C., M. Vidal, et al. (1995). "Truncated WT1 mutants alter the subnuclear localization of the wild-type protein." Proc Natl Acad Sci U S A **92**(26): 11960-4.

Era, T. (2002). "Bcr-Abl is a "molecular switch" for the decision for growth and differentiation in hematopoietic stem cells." Int J Hematol **76**(1): 35-43.

Farahat, N., A. Morilla, et al. (1998). "Detection of minimal residual disease in B-lineage acute lymphoblastic leukaemia by quantitative flow cytometry." Br J Haematol **101**(1): 158-64.

Fernandez-Luna, J. L. (2000). "Bcr-Abl and inhibition of apoptosis in chronic myelogenous leukemia cells." Apoptosis **5**(4): 315-8.

Ferrando, A. A. and A. T. Look (2000). "Clinical implications of recurring chromosomal and associated molecular abnormalities in acute lymphoblastic leukemia." Semin Hematol **37**(4): 381-95.

Forbes, L. V., R. E. Gale, et al. (2002). "An activating mutation in the transmembrane domain of the granulocyte colony-stimulating factor receptor in patients with acute myeloid leukemia." Oncogene **21**(39): 5981-9.

Foroni, L., C. J. Harrison, et al. (1999). "Investigation of minimal residual disease in childhood and adult acute lymphoblastic leukaemia by molecular analysis." Br J Haematol **105**(1): 7-24.

Fraizer, G. C., Y. J. Wu, et al. (1994). "Transcriptional regulation of the human Wilms' tumor gene (WT1). Cell type-specific enhancer and promiscuous promoter." J Biol Chem **269**(12): 8892-900.

Fujino, T., A. Suzuki, et al. (2002). "Single-translocation and double-chimeric transcripts: detection of NUP98-HOXA9 in myeloid leukemias with HOXA11 or HOXA13 breaks of the chromosomal translocation t(7;11)(p15;p15)." Blood **99**(4): 1428-33.

Gabert, J., E. Beillard, et al. (2003). "Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program." Leukemia **17**(12): 2318-57.

Gaiger, A., B. Linnerth, et al. (1999). "Wilms' tumour gene (wt1) expression at diagnosis has no prognostic relevance in childhood acute lymphoblastic leukaemia treated by an intensive chemotherapy protocol." Eur J Haematol **63**(2): 86-93.

Gaiger, A., V. Reese, et al. (2000). "Immunity to WT1 in the animal model and in patients with acute myeloid leukemia." Blood **96**(4): 1480-9.

Gaipa, G., G. Basso, et al. (2005). "Drug-induced immunophenotypic modulation in childhood ALL: implications for minimal residual disease detection." Leukemia. **19**(1): 49-56.

Gambacorti-Passerini, C. B., R. H. Gunby, et al. (2003). "Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias." Lancet Oncol **4**(2): 75-85.

Gameiro, P., I. Moreira, et al. (2002). "Polymerase chain reaction (PCR)- and reverse transcription PCR-based minimal residual disease detection in long-term follow-up of childhood acute lymphoblastic leukaemia." Br J Haematol **119**(3): 685-96.

Gao, L., I. Bellantuono, et al. (2000). "Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1." Blood **95**(7): 2198-203.

Garg, M., H. Moore, et al. (2003). "Prognostic significance of quantitative analysis of WT1 gene transcripts by competitive reverse transcription polymerase chain reaction in acute leukaemia." Br J Haematol **123**(1): 49-59.

Georgopoulos, K. (2002). "Haematopoietic cell-fate decisions, chromatin regulation and ikaros." Nat Rev Immunol **2**(3): 162-74.

Germano, G., L. del Giudice, et al. (2003). "Clonality profile in relapsed precursor-B-ALL children by GeneScan and sequencing analyses. Consequences on minimal residual disease monitoring." Leukemia **17**(8): 1573-82.

Giles, F. J., A. T. Stopeck, et al. (2003). "SU5416, a small molecule tyrosine kinase receptor inhibitor, has biologic activity in patients with refractory acute myeloid leukemia or myelodysplastic syndromes." Blood **20**: 20.

Gilliland, D. G. and J. D. Griffin (2002a). "The roles of FLT3 in hematopoiesis and leukemia." Blood **100**(5): 1532-42.

Gilliland, D. G. (2002b). "Molecular genetics of human leukemias: New insights into therapy." Semin Hematol **39**(4 Suppl 3): 6-11.

Goulden, N., A. Oakhill, et al. (2001). "Practical application of minimal residual disease assessment in childhood acute lymphoblastic leukaemia annotation." Br J Haematol **112**(2): 275-81.

Goulden, N. and C. Steward (2002). "Clinical relevance of MRD in children undergoing allogeneic stem cell transplantation for ALL." Best Pract Res Clin Haematol **15**(1): 59-70.

Goulden, N., P. Bader, et al. (2003). "Minimal residual disease prior to stem cell transplant for childhood acute lymphoblastic leukaemia." Br J Haematol **122**(1): 24-9.

Grabher, C., H. von Boehmer, et al. (2006). "Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia." Nat Rev Cancer. **6**(5): 347-59.

Graf Einsiedel, H., T. Taube, et al. (2001). "Prognostic value of p16(INK4a) gene deletions in pediatric acute lymphoblastic leukemia." Blood **97**(12): 4002-4.

Greaves, M. (2002). "Childhood leukaemia." Bmj **324**(7332): 283-7.

Greaves, M. F. and J. Wiemels (2003). "Origins of chromosome translocations in childhood leukaemia." Nat Rev Cancer **3**(9): 639-49.

Greaves, M. (2006). "Infection, immune responses and the aetiology of childhood leukaemia." Nat Rev Cancer **6**(3): 193-203.

Greiner, J., M. Ringhoffer, et al. (2004). "mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies." Int J Cancer **108**(5): 704-11.

Griesinger, F., M. Piro-Noack, et al. (1999). "Leukaemia-associated immunophenotypes (LAIP) are observed in 90% of adult and childhood acute lymphoblastic leukaemia: detection in remission marrow predicts outcome." Br J Haematol **105**(1): 241-55.

Griffin, J. (2001). "The biology of signal transduction inhibition: basic science to novel therapies." Semin Oncol **28**(5 Suppl 17): 3-8.

Grimwade, D. (1999). "The pathogenesis of acute promyelocytic leukaemia: evaluation of the role of molecular diagnosis and monitoring in the management of the disease." Br J Haematol **106**(3): 591-613.

Grimwade, D. (2001). "The clinical significance of cytogenetic abnormalities in acute myeloid leukaemia." Best Pract Res Clin Haematol **14**(3): 497-529.

Grimwade, D. (2002). "The significance of minimal residual disease in patients with t(15;17)." Best Pract Res Clin Haematol **15**(1): 137-58.

Grundler, R., C. Thiede, et al. (2003). "Sensitivity towards tyrosine kinase inhibitors varies between different activating mutations of the FLT3 receptor." Blood **27**: 27.

Guerrasio, A., C. Pilatrino, et al. (2002). "Assessment of minimal residual disease (MRD) in CBFbeta/MYH11-positive acute myeloid leukemias by qualitative and quantitative RT-PCR amplification of fusion transcripts." Leukemia **16**(6): 1176-81.

Guo, S. X., T. Taki, et al. (2000). "Hypermethylation of p16 and p15 genes and RB protein expression in acute leukemia." Leuk Res **24**(1): 39-46.

Guo, Y., H. Niiya, et al. (2005). "Direct recognition and lysis of leukemia cells by WT1-specific CD4+ T lymphocytes in an HLA class II-restricted manner." Blood. **106**(4): 1415-8. Epub 2005 Apr 21.

Haga, S. B., S. Fu, et al. (2000). "BP1, a new homeobox gene, is frequently expressed in acute leukemias." Leukemia **14**(11): 1867-75.

Hall, G. W. (2001). "Childhood myeloid leukaemias." Best Pract Res Clin Haematol **14**(3): 573-91.

Harigae, H. (2006). "GATA transcription factors and hematological diseases." Tohoku J Exp Med. **210**(1): 1-9.

Harrison, C. J. (2000). "The genetics of childhood acute lymphoblastic leukaemia." Baillieres Best Pract Res Clin Haematol **13**(3): 427-39.

Harrison, C. J. (2001). "Acute lymphoblastic leukaemia." Best Pract Res Clin Haematol **14**(3): 593-607.

Harrison, C. J. and L. Foroni (2002). "Cytogenetics and molecular genetics of acute lymphoblastic leukemia." Rev Clin Exp Hematol **6**(2): 91-113.

Hawley, R. G., A. Z. Fong, et al. (1997). "Transforming function of the HOX11/TCL3 homeobox gene." Cancer Res **57**(2): 337-45.

Hernandez-Boluda, J. C. and F. Cervantes (2002). "Imatinib mesylate (Gleevec, Glivec): a new therapy for chronic myeloid leukemia and other malignancies." Drugs Today (Barc) **38**(9): 601-13.

Hilden, J. M., J. L. Frestedt, et al. (1997). "Molecular analysis of infant acute leukemia." Leuk Lymphoma **25**(3-4): 191-9.

Hirose, M. (1999). "The role of Wilms' tumor genes." J Med Invest **46**(3-4): 130-40.

Hoelzer, D., N. Gokbuget, et al. (2002). "Acute lymphoblastic leukemia." Hematology: 162-92.

Hoffbrand, A., Lewis, SM, Tuddenham, EGD (1999). Postgraduate haematology. Oxford, Butterworth-Heinemann.

Hoffbrand, A., Pettit, JE, Moss, PAH (2001). Essential haematology. Oxford, Blackwell Science.

Hoffman, B., A. Amanullah, et al. (2002). "The proto-oncogene c-myc in hematopoietic development and leukemogenesis." Oncogene **21**(21): 3414-21.

Hofmann, W., H. D. Royer, et al. (1993). "Characterization of the transcriptional regulatory region of the human WT1 gene." Oncogene **8**(11): 3123-32.

Hosen, N., Y. Sonoda, et al. (2002). "Very low frequencies of human normal CD34+ haematopoietic progenitor cells express the Wilms' tumour gene WT1 at levels similar to those in leukaemia cells." Br J Haematol **116**(2): 409-20.

Hossain, A. and G. F. Saunders (2001). "The human sex-determining gene SRY is a direct target of WT1." J Biol Chem **276**(20): 16817-23. Epub 2001 Feb 20.

Hossain, A., M. Nixon, et al. (2006). "N-terminally truncated WT1 protein with oncogenic properties overexpressed in leukemia." J Biol Chem. **281**(38): 28122-30. Epub 2006 May 12.

Idelman, G., T. Glaser, et al. (2003). "WT1-p53 interactions in insulin-like growth factor-I receptor gene regulation." J Biol Chem **278**(5): 3474-82. Epub 2002 Nov 19.

Inokuchi, K., H. Yamaguchi, et al. (2002). "Loss of DCC gene expression is of prognostic importance in acute myelogenous leukemia." Clin Cancer Res **8**(6): 1882-8.

Inoue, K., H. Ogawa, et al. (1997). "Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia." Blood **89**(4): 1405-12.

Ito, S., Y. Ishida, et al. (2001). "Flow cytometric analysis of aberrant antigen expression of blasts using CD45 blast gating for minimal residual disease in acute leukemia and high-risk myelodysplastic syndrome." Leuk Res **25**(3): 205-11.

Ito, K., Y. Oji, et al. (2006). "Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway." Oncogene. **25**(30): 4217-29. Epub 2006 Mar 6.

Iwai, T., S. Yokota, et al. (1999). "Internal tandem duplication of the FLT3 gene and clinical evaluation in childhood acute myeloid leukemia. The Children's Cancer and Leukemia Study Group, Japan." Leukemia **13**(1): 38-43.

Izon, D. J., J. A. Punt, et al. (2002). "Deciphering the role of Notch signaling in lymphopoiesis." Curr Opin Immunol **14**(2): 192-9.

Jilani, I., E. Estey, et al. (2003). "Better detection of FLT3 internal tandem duplication using peripheral blood plasma DNA." Leukemia **17**(1): 114-9.

Kainz, B., D. Heintel, et al. (2002). "Variable prognostic value of FLT3 internal tandem duplications in patients with de novo AML and a normal karyotype, t(15;17), t(8;21) or inv(16)." Hematol J **3**(6): 283-9.

Kaleem, Z., E. Crawford, et al. (2003). "Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data." Arch Pathol Lab Med **127**(1): 42-8.

Kalina, T., M. Vaskova, et al. (2005). "Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of CD66c distinct from other myeloid antigens." BMC Cancer. **5**(1): 38.

Kaneuchi, M., M. Sasaki, et al. (2005). "WT1 and WT1-AS genes are inactivated by promoter methylation in ovarian clear cell adenocarcinoma." Cancer. **104**(9): 1924-30.

Karakas, T., C. C. Miething, et al. (2002). "The coexpression of the apoptosis-related genes bcl-2 and wt1 in predicting survival in adult acute myeloid leukemia." Leukemia **16**(5): 846-54.

Kawagoe, H., R. K. Humphries, et al. (1999). "Expression of HOX genes, HOX cofactors, and MLL in phenotypically and functionally defined subpopulations of leukemic and normal human hematopoietic cells." Leukemia **13**(5): 687-98.

Kawagoe, H., R. Kawagoe, et al. (2001). "Targeted down-regulation of MLL-AF9 with antisense oligodeoxyribonucleotide reduces the expression of the HOXA7 and -A10 genes and induces apoptosis in a human leukemia cell line, THP-1." Leukemia **15**(11): 1743-9.

Kawamata, S., C. Du, et al. (2002). "Overexpression of the Notch target genes Hes in vivo induces lymphoid and myeloid alterations." Oncogene **21**(24): 3855-63.

Keilholz, U., H. D. Menssen, et al. (2005). "Wilms' tumour gene 1 (WT1) in human neoplasia." Leukemia. **19**(8): 1318-23.

Kelly, L. M. and D. G. Gilliland (2002). "Genetics of myeloid leukemias." Annu Rev Genomics Hum Genet **3**: 179-98.

Kern, W., S. Danhauser-Riedl, et al. (2003). "Detection of minimal residual disease in unselected patients with acute myeloid leukemia using multiparameter flow cytometry for definition of leukemia-associated immunophenotypes and determination of their frequencies in normal bone marrow." Haematologica **88**(6): 646-53.

Kerst, G., H. Kreyenberg, et al. (2005). "Concurrent detection of minimal residual disease (MRD) in childhood acute lymphoblastic leukaemia by flow cytometry and real-time PCR." Br J Haematol. **128**(6): 774-82.

Khalidi, H. S., L. J. Medeiros, et al. (1998). "The immunophenotype of adult acute myeloid leukemia: high frequency of lymphoid antigen expression and comparison of immunophenotype, French-American-British classification, and karyotypic abnormalities." Am J Clin Pathol **109**(2): 211-20.

Kim, S. W., H. Y. Kwon, et al. (2003). "Reversal of P-glycoprotein-mediated multidrug resistance by ginsenoside Rg(3)." Biochem Pharmacol **65**(1): 75-82.

King-Underwood, L. and K. Pritchard-Jones (1998). "Wilms' tumor (WT1) gene mutations occur mainly in acute myeloid leukemia and may confer drug resistance." Blood **91**(8): 2961-8.

Kitagawa, Y., K. Inoue, et al. (2002). "Prevalent involvement of illegitimate V(D)J recombination in chromosome 9p21 deletions in Lymphoid Leukemia." J Biol Chem **12**: 12.

Kitareewan, S., I. Pitha-Rowe, et al. (2002). "UBE1L is a retinoid target that triggers PML/RARalpha degradation and apoptosis in acute promyelocytic leukemia." Proc Natl Acad Sci U S A **99**(6): 3806-11.

Kiyoi, H., T. Naoe, et al. (1999). "Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia." Blood **93**(9): 3074-80.

Kiyoi, H. and T. Naoe (2002). "FLT3 in human hematologic malignancies." Leuk Lymphoma **43**(8): 1541-7.

Kletzel, M., M. Olzewski, et al. (2002). "Utility of WT1 as a reliable tool for the detection of minimal residual disease in children with leukemia." Pediatr Dev Pathol **5**(3): 269-75.

Knechtli, C. J., N. J. Goulden, et al. (1998a). "Minimal residual disease status before allogeneic bone marrow transplantation is an important determinant of successful outcome for children and adolescents with acute lymphoblastic leukemia." Blood **92**(11): 4072-9.

Knechtli, C. J., N. J. Goulden, et al. (1998b). "Minimal residual disease status as a predictor of relapse after allogeneic bone marrow transplantation for children with acute lymphoblastic leukaemia." Br J Haematol **102**(3): 860-71.

Knowles, D. (2001). Neoplastic hematopathology. Philadelphia, Lippincott Williams & Wilkins.

Kojika, S. and J. D. Griffin (2001). "Notch receptors and hematopoiesis." Exp Hematol **29**(9): 1041-52.

Kondo, M., K. Horibe, et al. (1999). "Prognostic value of internal tandem duplication of the FLT3 gene in childhood acute myelogenous leukemia." Med Pediatr Oncol **33**(6): 525-9.

Kottaridis, P. D., R. E. Gale, et al. (2001). "The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials." Blood **98**(6): 1752-9.

Kottaridis, P. D., R. E. Gale, et al. (2002). "Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors." Blood **100**(7): 2393-8.

Kottaridis, P. D., R. E. Gale, et al. (2003). "Prognostic implications of the presence of FLT3 mutations in patients with acute myeloid leukemia." Leuk Lymphoma **44**(6): 905-13.

Kreuzer, K. A., A. Saborowski, et al. (2001). "Fluorescent 5'-exonuclease assay for the absolute quantification of Wilms' tumour gene (WT1) mRNA: implications for monitoring human leukaemias." Br J Haematol **114**(2): 313-8.

Krug, U., A. Ganser, et al. (2002). "Tumor suppressor genes in normal and malignant hematopoiesis." Oncogene **21**(21): 3475-95.

Kuerbitz, S. J., J. Pahys, et al. (2002). "Hypermethylation of the imprinted NNAT locus occurs frequently in pediatric acute leukemia." Carcinogenesis **23**(4): 559-64.

Kundu, M. and P. P. Liu (2001). "Function of the inv(16) fusion gene CBFB-MYH11." Curr Opin Hematol **8**(4): 201-5.

La Starza, R., A. Vitale, et al. (2002). "Philadelphia-positive acute lymphoblastic leukemia with multiple subclones including duplication of the Philadelphia chromosome and Abelson oncogene." Cancer Genet Cytogenet **132**(1): 46-50.

La Starza, R., M. Trubia, et al. (2003). "Human homeobox gene HOXC13 is the partner of NUP98 in adult acute myeloid leukemia with t(11;12)(p15;q13)." Genes Chromosomes Cancer **36**(4): 420-3.

Laczika, K., G. Mitterbauer, et al. (2001). "Prospective monitoring of minimal residual disease in acute myeloid leukemia with inversion(16) by CBFbeta/MYH11 RT-PCR: implications for a monitoring schedule and for treatment decisions." Leuk Lymphoma **42**(5): 923-31.

Ladomery, M. R., J. Slight, et al. (1999). "Presence of WT1, the Wilm's tumor suppressor gene product, in nuclear poly(A)(+) ribonucleoprotein." J Biol Chem **274**(51): 36520-6.

Langerak, A. W., T. Szczepanski, et al. (1997). "Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations." Leukemia. **11**(12): 2192-9.

Langerak, A. W., I. L. Wolvers-Tettero, et al. (2001). "Basic helix-loop-helix proteins E2A and HEB induce immature T-cell receptor rearrangements in nonlymphoid cells." Blood. **98**(8): 2456-65.

Lapillonne, H., A. Renneville, et al. (2006). "High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia." J Clin Oncol. **24**(10): 1507-15.

Larson, R. A. (2003). "Is modulation of multidrug resistance a viable strategy for acute myeloid leukemia?" Leukemia **17**(3): 488-91.

Lawrence, H. J., S. Rozenfeld, et al. (1999). "Frequent co-expression of the HOXA9 and MEIS1 homeobox genes in human myeloid leukemias." Leukemia **13**(12): 1993-9.

Lee, S. B. and D. A. Haber (2001). "Wilms tumor and the WT1 gene." Exp Cell Res **264**(1): 74-99.

Legrand, O., J. Y. Perrot, et al. (2000). "The immunophenotype of 177 adults with acute myeloid leukemia: proposal of a prognostic score." Blood **96**(3): 870-7.

Levis, M., J. Allebach, et al. (2002). "A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo." Blood **99**(11): 3885-91.

Li, A. H., R. Rosenquist, et al. (2001). "Detailed clonality analysis of relapsing precursor B acute lymphoblastic leukemia: implications for minimal residual disease detection." Leuk Res **25**(12): 1033-45.

Liang, D. C., L. Y. Shih, et al. (2002). "Clinical relevance of internal tandem duplication of the FLT3 gene in childhood acute myeloid leukemia." Cancer **94**(12): 3292-8.

Liang, D. C., L. Y. Shih, et al. (2003). "FLT3-TKD mutation in childhood acute myeloid leukemia." Leukemia **17**(5): 883-6.

Lin, T. S., S. Mahajan, et al. (2000). "STAT signaling in the pathogenesis and treatment of leukemias." Oncogene **19**(21): 2496-504.

Little, N. A., N. D. Hastie, et al. (2000). "Identification of WTAP, a novel Wilms' tumour 1-associating protein." Hum Mol Genet **9**(15): 2231-9.

Liu Yin, J. A. (2002). "Minimal residual disese in acute myeloid leukaemia." Best Pract Res Clin Haematol **15**(1): 119-135.

Lo Coco, F., D. Diverio, et al. (1999). "Genetic diagnosis and molecular monitoring in the management of acute promyelocytic leukemia." Blood **94**(1): 12-22.

Lo-Coco, F., M. Breccia, et al. (2003a). "Diagnostic value of detecting fusion proteins derived from chromosome translocations in acute leukaemia." Best Pract Res Clin Haematol **16**(4): 653-70.

Lo-Coco, F., M. Breccia, et al. (2003b). "The importance of molecular monitoring in acute promyelocytic leukaemia." Best Pract Res Clin Haematol **16**(3): 503-20.

Loeb, D. M., E. Evron, et al. (2001). "Wilms' tumor suppressor gene (WT1) is expressed in primary breast tumors despite tumor-specific promoter methylation." Cancer Res. **61**(3): 921-5.

Loeb, D. M., D. Korz, et al. (2002a). "Cyclin E is a target of WT1 transcriptional repression." J Biol Chem **277**(22): 19627-32.

Loeb, D. M. and S. Sukumar (2002b). "The role of WT1 in oncogenesis: tumor suppressor or oncogene?" Int J Hematol. **76**(2): 117-26.

Lorsbach, R. B. and J. R. Downing (2001). "The role of the AML1 transcription factor in leukemogenesis." Int J Hematol **74**(3): 258-65.

Lucio, P., A. Parreira, et al. (1999). "Flow cytometric analysis of normal B cell differentiation: a frame of reference for the detection of minimal residual disease in precursor-B-ALL." Leukemia **13**(3): 419-27.

Lucio, P., G. Gaipa, et al. (2001). "BIOMED-I concerted action report: flow cytometric immunophenotyping of precursor B-ALL with standardized triple-stainings. BIOMED-1 Concerted Action Investigation of Minimal Residual Disease in Acute Leukemia: International Standardization and Clinical Evaluation." Leukemia **15**(8): 1185-92.

Magli, M. C., C. Largman, et al. (1997). "Effects of HOX homeobox genes in blood cell differentiation." J Cell Physiol **173**(2): 168-77.

Magyarosy, E., N. Varga, et al. (2003). "Follow-up of minimal residual disease in acute childhood lymphoblastic leukemia by WT1 gene expression in the peripheral blood: the Hungarian experience." Pediatr Hematol Oncol **20**(1): 65-74.

Malaska, J., M. Sklenickova, et al. (2000). "Telomerase activity and expression and telomere analysis in situ in the course of treatment of childhood leukemias." Blood Cells Mol Dis **26**(5): 534-9.

Malec, M., E. Bjorklund, et al. (2001). "Flow cytometry and allele-specific oligonucleotide PCR are equally effective in detection of minimal residual disease in ALL." Leukemia **15**(5): 716-27.

Malec, M., V. H. van der Velden, et al. (2004). "Analysis of minimal residual disease in childhood acute lymphoblastic leukemia: comparison between RQ-PCR analysis of Ig/TcR gene rearrangements and multicolor flow cytometric immunophenotyping." Leukemia. **18**(10): 1630-6.

Malik, K., A. Salpekar, et al. (2000). "Identification of differential methylation of the WT1 antisense regulatory region and relaxation of imprinting in Wilms' tumor." Cancer Res. **60**(9): 2356-60.

Marcucci, G., M. A. Caligiuri, et al. (2001). "Quantification of CBFbeta/MYH11 fusion transcript by real time RT-PCR in patients with INV(16) acute myeloid leukemia." Leukemia **15**(7): 1072-80.

Mares, J., V. Kriz, et al. (2001). "Methylation changes in promoter and enhancer regions of the WT1 gene in Wilms' tumours." Cancer Lett. **166**(2): 165-71.

Markaki, E. A., M. Tsopanomichalou, et al. (2001). "Mutations of retinoblastoma gene (Rb-1) as a prognostic factor in children with acute leukemia and neuroblastoma." Pediatr Hematol Oncol **18**(2): 101-10.

Marshall, G. M., M. Haber, et al. (2003). "Importance of minimal residual disease testing during the second year of therapy for children with acute lymphoblastic leukemia." J Clin Oncol **21**(4): 704-9.

Matsushita, M., H. Ikeda, et al. (2001). "Quantitative monitoring of the PRAME gene for the detection of minimal residual disease in leukaemia." Br J Haematol **112**(4): 916-26.

Mattsson, K., K. Pokrovskaja, et al. (2001). "Proteins associated with the promyelocytic leukemia gene product (PML)-containing nuclear body move to the nucleolus upon inhibition of proteasome-dependent protein degradation." Proc Natl Acad Sci U S A **98**(3): 1012-7.

Maurer, U., E. Weidmann, et al. (1997). "Wilms tumor gene (wt1) mRNA is equally expressed in blast cells from acute myeloid leukemia and normal CD34+ progenitors." Blood **90**(10): 4230-2.

McKenna, R. W., L. T. Washington, et al. (2001). "Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry." Blood **98**(8): 2498-507.

McKinnell, R., Parchment, RE, Perantoni, AO, Pierce, GB (1998). The biological basis of cancer. Cambridge, Cambridge University Press.

Menke, A. L., A. R. Clarke, et al. (2002). "Genetic interactions between the Wilms' tumor 1 gene and the p53 gene." Cancer Res **62**(22): 6615-20.

Menssen, H. D., H. J. Renkl, et al. (1995). "Presence of Wilms' tumor gene (wt1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias." Leukemia **9**(6): 1060-7.

Menssen, H. D., H. J. Renkl, et al. (1997). "Detection by monoclonal antibodies of the Wilms' tumor (WT1) nuclear protein in patients with acute leukemia." Int J Cancer **70**(5): 518-23.

Menssen, H. D., J. M. Siehl, et al. (2002). "Wilms tumor gene (WT1) expression as a panleukemic marker." Int J Hematol **76**(2): 103-9.

Meshinchi, S., W. G. Woods, et al. (2001). "Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia." Blood **97**(1): 89-94.

Meshinchi, S., D. L. Stirewalt, et al. (2003). "Activating mutations of RTK/ras signal transduction pathway in pediatric acute myeloid leukemia." Blood **102**(4): 1474-9.

Metzler, M., U. Brehm, et al. (2004). "Asymmetric multiplex-polymerase chain reaction - a high throughput method for detection and sequencing genomic fusion sites in t(4;11)." Br J Haematol **124**(1): 47-54.

Millard, I., E. Degrave, et al. (1998). "Detection of intracellular antigens by flow cytometry: comparison of two chemical methods and microwave heating." Clin Chem **44**(11): 2320-30.

Milner, L. A. and A. Bigas (1999). "Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation." Blood **93**(8): 2431-48.

Moorwood, K., A. Salpekar, et al. (1999). "Transactivation of the WT1 antisense promoter is unique to the WT1[+/-] isoform." FEBS Lett **456**(1): 131-6.

Moppett, J., G. A. Burke, et al. (2003). "The clinical relevance of detection of minimal residual disease in childhood acute lymphoblastic leukaemia." J Clin Pathol **56**(4): 249-53.

Moreno, I., G. Martin, et al. (2003). "Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia." Haematologica **88**(1): 19-24.

Morschhauser, F., J. M. Cayuela, et al. (2000). "Evaluation of minimal residual disease using reverse-transcription polymerase chain reaction in t(8;21) acute myeloid leukemia: a multicenter study of 51 patients." J Clin Oncol **18**(4): 788-94.

Munoz, L., O. Lopez, et al. (2000). "Combined use of reverse transcriptase polymerase chain reaction and flow cytometry to study minimal residual disease in Philadelphia positive acute lymphoblastic leukemia." Haematologica **85**(7): 704-10.

Muntean, A. G., Y. Ge, et al. (2006). "Transcription factor GATA-1 and Down syndrome leukemogenesis." Leuk Lymphoma. **47**(6): 986-97.

Murphy, K. M., M. Levis, et al. (2003). "Detection of FLT3 Internal Tandem Duplication and D835 Mutations by a Multiplex Polymerase Chain Reaction and Capillary Electrophoresis Assay." J Mol Diagn **5**(2): 96-102.

Nagler, A., R. Condiotti, et al. (1999). "Detection of minimal residual disease (MRD) after bone marrow transplantation (BMT) by multi-parameter flow cytometry (MPFC)." Med Oncol **16**(3): 177-87.

Nakatsuka, S., Y. Oji, et al. (2006). "Immunohistochemical detection of WT1 protein in a variety of cancer cells." Mod Pathol. **19**(6): 804-14.

Nakamura, T., Y. Yamazaki, et al. (1999). "NUP98 is fused to PMX1 homeobox gene in human acute myelogenous leukemia with chromosome translocation t(1;11)(q23;p15)." Blood **94**(2): 741-7.

Neale, G. A., E. Coustan-Smith, et al. (1999). "Tandem application of flow cytometry and polymerase chain reaction for comprehensive detection of minimal residual disease in childhood acute lymphoblastic leukemia." Leukemia **13**(8): 1221-6.

Neale, G. A., D. Campana, et al. (2003). "Minimal residual disease detection in acute lymphoblastic leukemia: real improvement with the real-time quantitative PCR method?" J Pediatr Hematol Oncol **25**(2): 100-2.

Neale, G. A., E. Coustan-Smith, et al. (2004). "Comparative analysis of flow cytometry and polymerase chain reaction for the detection of minimal residual disease in childhood acute lymphoblastic leukemia." Leukemia **18**(5): 934-8.

Neglia, J. P., A. T. Meadows, et al. (1991). "Second neoplasms after acute lymphoblastic leukemia in childhood." N Engl J Med **325**(19): 1330-6.

Niegemann, E., S. Wehner, et al. (1999). "wt1 gene expression in childhood leukemias." Acta Haematol **102**(2): 72-6.

Niksic, M., J. Slight, et al. (2004). "The Wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes." Hum Mol Genet **13**(4): 463-71. Epub 2003 Dec 17.

Nishida, S., N. Hosen, et al. (2006). "AML1-ETO rapidly induces acute myeloblastic leukemia in cooperation with the Wilms tumor gene, WT1." Blood. **107**(8): 3303-12. Epub 2005 Dec 27.

Noguera, N. I., M. Breccia, et al. (2002). "Alterations of the FLT3 gene in acute promyelocytic leukemia: association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol." Leukemia **16**(11): 2185-9.

Nunez, C. A., T. F. Zipf, et al. (2003). "Molecular monitoring of cerebrospinal fluid can predict clinical relapse in acute lymphoblastic leukemia with eosinophilia." Arch Pathol Lab Med **127**(5): 601-5.

Nyvold, C., H. O. Madsen, et al. (2002). "Precise quantification of minimal residual disease at day 29 allows identification of children with acute lymphoblastic leukemia and an excellent outcome." Blood **99**(4): 1253-8.

Oelschlagel, U., R. Nowak, et al. (2000). "Shift of aberrant antigen expression at relapse or at treatment failure in acute leukemia." Cytometry **42**(4): 247-53.

O'Farrell, A. M., T. J. Abrams, et al. (2003). "SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo." Blood **101**(9): 3597-605.

Ogawa, M. and T. Matsunaga (1999). "Humoral regulation of hematopoietic stem cells." Ann N Y Acad Sci **872**: 17-23.

Ogawa, H., H. Tamaki, et al. (2003). "The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia." Blood **101**(5): 1698-704.

Oka, Y., A. Tsuboi, et al. (2002). "WT1 as a novel target antigen for cancer immunotherapy." Curr Cancer Drug Targets **2**(1): 45-54.

Oka, Y., A. Tsuboi, et al. (2003). "Wilms tumor gene peptide-based immunotherapy for patients with overt leukemia from myelodysplastic syndrome (MDS) or MDS with myelofibrosis." Int J Hematol **78**(1): 56-61.

Oka, Y., A. Tsuboi, et al. (2004). "Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression." Proc Natl Acad Sci U S A **101**(38): 13885-90. Epub 2004 Sep 13.

Olivero, S., C. Maroc, et al. (2000). "Detection of different Ikaros isoforms in human leukaemias using real-time quantitative polymerase chain reaction." Br J Haematol **110**(4): 826-30.

Olszewski, M., W. Huang, et al. (2005). "Wilms' tumor 1 (WT1) gene in hematopoiesis: a surrogate marker of cell proliferation as a possible mechanism of action?" Cytotherapy. **7**(1): 57-61.

Orfao, A., M. C. Chillon, et al. (1999). "The flow cytometric pattern of CD34, CD15 and CD13 expression in acute myeloblastic leukemia is highly characteristic of the presence of PML-RARalpha gene rearrangements." Haematologica **84**(5): 405-12.

Ormerod, M. G. (2000). Flow Cytometry. Oxford, Oxford University Press.

Osborne, D., L. Frost, et al. (2005). "Elevated levels of WT1 transcripts in bone marrow harvests are associated with a high relapse risk in patients autografted for acute myeloid leukaemia." Bone Marrow Transplant. **36**(1): 67-70.

Ostergaard, M., L. H. Olesen, et al. (2004). "WT1 gene expression: an excellent tool for monitoring minimal residual disease in 70 % of acute myeloid leukaemia patients - results from a single-centre study." Br J Haematol **125**(5): 590-600.

Otten, J., N. Philippe, et al. (2002). "The Children Leukemia Group: 30 years of research and achievements." Eur J Cancer **38**(4): S44-9.

Owens, M. A., H. G. Vall, et al. (2000). "Validation and quality control of immunophenotyping in clinical flow cytometry." J Immunol Methods **243**(1-2): 33-50.

Owens, B. M. and R. G. Hawley (2002). "HOX and non-HOX omeobox genes in leukemic hematopoiesis." Stem Cells **20**(5): 364-79.

Ozgen, U., S. Anak, et al. (2000). "wt1 gene expression in childhood acute leukemias." Acta Haematol **103**(4): 229-30.

Pallis, M., J. Turzanski, et al. (2003). "Resistance to spontaneous apoptosis in acute myeloid leukaemia blasts is associated with p-glycoprotein expression and function, but not with the presence of FLT3 internal tandem duplications." Br J Haematol **120**(6): 1009-16.

Pane, F., M. Intrieri, et al. (2002). "BCR/ABL genes and leukemic phenotype: from molecular mechanisms to clinical correlations." Oncogene **21**(56): 8652-67.

Panzer-Grumayer, E. R., M. Schneider, et al. (2000). "Rapid molecular response during early induction chemotherapy predicts a good outcome in childhood acute lymphoblastic leukemia." Blood **95**(3): 790-4.

Perentesis, J. P. and E. L. Sievers (2001). "Targeted therapies for high-risk acute myeloid leukemia." Hematol Oncol Clin North Am **15**(4): 677-701, viii-ix.

Perrotti, D., V. Cesi, et al. (2002). "BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2." Nat Genet **30**(1): 48-58.

Perry, C., A. Eldor, et al. (2002). "Runx1/AML1 in leukemia: disrupted association with diverse protein partners." Leuk Res **26**(3): 221-8.

Pine, S. R., F. H. Moy, et al. (2003). "Real-time quantitative PCR: standardized detection of minimal residual disease in pediatric acute lymphoblastic leukemia. Polymerase chain reaction." J Pediatr Hematol Oncol **25**(2): 103-8.

Pongers-Willemse, M. J., O. J. Verhagen, et al. (1998). "Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes." Leukemia. **12**(12): 2006-14.

Pongers-Willemse, M. J., T. Seriu, et al. (1999). "Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the BIOMED-1 CONCERTED ACTION: investigation of minimal residual disease in acute leukemia." Leukemia **13**(1): 110-8.

Porwit-MacDonald, A., E. Bjorklund, et al. (2000). "BIOMED-1 concerted action report: flow cytometric characterization of CD7+ cell subsets in normal bone marrow as a basis for the diagnosis and follow-up of T cell acute lymphoblastic leukemia (T-ALL)." Leukemia **14**(5): 816-25.

Preudhomme, C., C. Sagot, et al. (2002). "Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA)." Blood **100**(8): 2717-23.

Pui, C. H. (2000). "Acute lymphoblastic leukemia in children." Curr Opin Oncol **12**(1): 3-12.

Pui, C. H., D. Campana, et al. (2001). "Childhood acute lymphoblastic leukaemia--current status and future perspectives." Lancet Oncol **2**(10): 597-607.

Qiao, Y., S. Ogawa, et al. (2003). "Identification of a novel fusion gene, TTL, fused to ETV6 in acute lymphoblastic leukemia with t(12;13)(p13;q14), and its implication in leukemogenesis." Leukemia **17**(6): 1112-20.

Ramirez, O., A. Linares, et al. (2003). "WT1 mRNA in cerebrospinal fluid associated with relapse in pediatric lymphoblastic leukemia." J Pediatr Hematol Oncol **25**(6): 453-8.

Ravandi, F., M. Talpaz, et al. (2002). "Cellular signalling pathways: new targets in leukaemia therapy." Br J Haematol **116**(1): 57-77.

Ravindranath, Y. (2003). "Recent advances in pediatric acute lymphoblastic and myeloid leukemia." Curr Opin Oncol **15**(1): 23-35.

Rebollo, A. and A. C. Martinez (1999). "Ras proteins: recent advances and new functions." Blood **94**(9): 2971-80.

Reichle, A., G. Rothe, et al. (1999). "Transplant characteristics: minimal residual disease and impaired megakaryocytic colony growth as sensitive parameters for predicting relapse in acute myeloid leukemia." Leukemia **13**(8): 1227-34.

Reilly, J. T. (2003). "FLT3 and its role in the pathogenesis of acute myeloid leukaemia." Leuk Lymphoma **44**(1): 1-7.

Reinhardt, D., C. Langebrake, et al. (2002). "[Minimal residual disease in acute myeloid leukemia in children--standardization and evaluation of immunophenotyping in the AML-BFM-98 study]." Klin Padiatr **214**(4): 179-87.

Renshaw, J., L. King-Underwood, et al. (1997). "Differential splicing of exon 5 of the Wilms tumour (WTI) gene." Genes Chromosomes Cancer **19**(4): 256-66.

Rizzatti, E. G., A. B. Garcia, et al. (2002). "Expression of CD117 and CD11b in bone marrow can differentiate acute promyelocytic leukemia from recovering benign myeloid proliferation." Am J Clin Pathol **118**(1): 31-7.

Rosenfeld, C., M. A. Cheever, et al. (2003). "WT1 in acute leukemia, chronic myelogenous leukemia and myelodysplastic syndrome: therapeutic potential of WT1 targeted therapies." Leukemia **17**(7): 1301-12.

Roumier, C., P. Fenaux, et al. (2003). "New mechanisms of AML1 gene alteration in hematological malignancies." Leukemia **17**(1): 9-16.

Rubnitz, J. E., S. C. Raimondi, et al. (2002). "Characteristics and outcome of t(8;21)-positive childhood acute myeloid leukemia: a single institution's experience." Leukemia **16**(10): 2072-7.

San Miguel, J. F., M. B. Vidriales, et al. (2001). "Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification." Blood **98**(6): 1746-51.

San-Miguel, J. F., M. B. Vidriales, et al. (2002). "Immunological evaluation of minimal residual disease (MRD) in acute myeloid leukaemia (AML)." Best Pract Res Clin Haematol **15**(1): 105-18.

Sanchez, J., J. Serrano, et al. (2002). "Clinical value of immunological monitoring of minimal residual disease in acute lymphoblastic leukaemia after allogeneic transplantation." Br J Haematol **116**(3): 686-94.

Sang, B. C., L. Shi, et al. (1997). "Monoclonal antibodies specific to the acute lymphoblastic leukemia t(1;19)-associated E2A/pbx1 chimeric protein: characterization and diagnostic utility." Blood **89**(8): 2909-14.

Satoh, Y., T. Nakagawachi, et al. (2003). "Significant reduction of WT1 gene expression, possibly due to epigenetic alteration in Wilms' tumor." J Biochem (Tokyo). **133**(3): 303-8.

Sattler, M., E. Pisick, et al. (2000). "Role of the cytoskeletal protein paxillin in oncogenesis." Crit Rev Oncog **11**(1): 63-76.

Sattler, M. and J. D. Griffin (2001). "Mechanisms of transformation by the BCR/ABL oncogene." Int J Hematol **73**(3): 278-91.

Sauerbrey, A., W. Sell, et al. (2002). "Expression of the BCRP gene (ABCG2/MXR/ABCP) in childhood acute lymphoblastic leukaemia." Br J Haematol **118**(1): 147-50.

Scharnhorst, V., P. Dekker, et al. (1999). "Internal translation initiation generates novel WT1 protein isoforms with distinct biological properties." J Biol Chem **274**(33): 23456-62.

Scharnhorst, V., A. J. van der Eb, et al. (2001). "WT1 proteins: functions in growth and differentiation." Gene **273**(2): 141-61.

Scheibenbogen, C., A. Letsch, et al. (2002). "CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia." Blood **100**(6): 2132-7.

Scheijen, B. and J. D. Griffin (2002). "Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease." Oncogene **21**(21): 3314-33.

Schmid, D., G. Heinze, et al. (1997). "Prognostic significance of WT1 gene expression at diagnosis in adult de novo acute myeloid leukemia." Leukemia **11**(5): 639-43.

Schmitt, C., C. Tonnelle, et al. (2002). "Aiolos and Ikaros: regulators of lymphocyte development, homeostasis and lymphoproliferation." Apoptosis **7**(3): 277-84.

Schnittger, S., C. Schoch, et al. (2002). "Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease." Blood **100**(1): 59-66.

Schrappe, M., R. Beier, et al. (2002). "New treatment strategies in childhood acute lymphoblastic leukaemia." Best Pract Res Clin Haematol **15**(4): 729-40.

Scolnik, M. P., R. Morilla, et al. (2002). "CD34 and CD117 are overexpressed in AML and may be valuable to detect minimal residual disease." Leuk Res **26**(7): 615-9.

Scrideli, C. A., S. Kashima, et al. (2002). "Minimal residual disease in Brazilian children with acute lymphoid leukemia: comparison of three detection methods by PCR." Leuk Res **26**(5): 431-8.

Shah, S. J., J. W. Taub, et al. (2001). "Relationship of p15 and p16 gene alterations to elevated dihydrofolate reductase in childhood acute lymphoblastic leukaemia." Br J Haematol **113**(3): 746-56.

Shao, Y., J. Lu, et al. (2005). "Histone acetyltransferase p300 promotes the activation of human WT1 promoter and intronic enhancer." Arch Biochem Biophys. **436**(1): 62-8.

Shih, L. Y., C. F. Huang, et al. (2002). "Internal tandem duplication of FLT3 in relapsed acute myeloid leukemia: a comparative analysis of bone marrow samples from 108 adult patients at diagnosis and relapse." Blood **100**(7): 2387-92.

Shimamoto, T., K. Ohyashiki, et al. (1998). "Homeobox genes in hematopoiesis and leukemogenesis." Int J Hematol **67**(4): 339-50.

Shusterman, S. and A. T. Meadows (2000). "Long term survivors of childhood leukemia." Curr Opin Hematol **7**(4): 217-22.

Siehl, J. M., E. Thiel, et al. (2002). "Quantitative real-time RT-PCR detects elevated Wilms tumor gene (WT1) expression in autologous blood stem cell preparations (PBSCs) from acute myeloid leukemia (AML) patients indicating contamination with leukemic blasts." Bone Marrow Transplant **29**(5): 379-81.

Siehl, J. M., E. Thiel, et al. (2003). "Possible regulation of Wilms' tumour gene 1 (WT1) expression by the paired box genes PAX2 and PAX8 and by the haematopoietic transcription factor GATA-1 in human acute myeloid leukaemias." Br J Haematol **123**(2): 235-42.

Siehl, J. M., M. Reinwald, et al. (2004). "Expression of Wilms' tumor gene 1 at different stages of acute myeloid leukemia and analysis of its major splice variants." Ann Hematol **31**(2): 31.

Sievers, E. L., B. J. Lange, et al. (2003). "Immunophenotypic evidence of leukemia after induction therapy predicts relapse: results from a prospective Children's Cancer Group study of 252 patients with acute myeloid leukemia." Blood **101**(9): 3398-406.

Silberstein, G. B., K. Van Horn, et al. (1997). "Altered expression of the WT1 wilms tumor suppressor gene in human breast cancer." Proc Natl Acad Sci U S A **94**(15): 8132-7.

Singh, N., R. A. Phillips, et al. (2000). "Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis." Exp Hematol **28**(5): 527-34.

Skorski, T. (2002). "BCR/ABL regulates response to DNA damage: the role in resistance to genotoxic treatment and in genomic instability." Oncogene **21**(56): 8591-604.

Spiekermann, K., K. Bagrintseva, et al. (2002). "A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia." Blood **100**(9): 3423-5.

Stamatoyannopoulos, G., Majerus, PW, Perlmutter, RM, Varmus, H (2001). The molecular basis of blood diseases. Philadelphia, W.B. Saunders Company.

Steinbach, D., W. Sell, et al. (2002a). "BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia." Leukemia **16**(8): 1443-7.

Steinbach, D., J. Hermann, et al. (2002b). "Clinical implications of PRAME gene expression in childhood acute myeloid leukemia." Cancer Genet Cytogenet **133**(2): 118-23.

Steinbach, D., S. Furchtbar, et al. (2003). "Contrary to adult patients, expression of the multidrug resistance gene (MDR1) fails to define a poor prognostic group in childhood AML." Leukemia **17**(2): 470-1.

Stirewalt, D. L., C. L. Willman, et al. (2001). "Quantitative, real-time polymerase chain reactions for FLT3 internal tandem duplications are highly sensitive and specific." Leuk Res **25**(12): 1085-8.

Stirewalt, D. L. and J. P. Radich (2003). "The role of FLT3 in haematopoietic malignancies." Nat Rev Cancer **3**(9): 650-65.

Sugita, K., T. Mori, et al. (1999). "The KOR-SA3544 antigen predominantly expressed on the surface of Philadelphia chromosome-positive acute lymphoblastic leukemia cells is nonspecific cross-reacting antigen-50/90 (CD66c) and invariably expressed in cytoplasm of human leukemia cells." Leukemia **13**(5): 779-85.

Sugiyama, H. (1998). "Wilms tumor gene (WT1) as a new marker for the detection of minimal residual disease in leukemia." Leuk Lymphoma **30**(1-2): 55-61.

Sugiyama, H. (2001). "Wilms' tumor gene WT1: its oncogenic function and clinical application." Int J Hematol **73**(2): 177-87.

Sugiyama, H. (2002). "Wilms tumor gene WT1 as a tumor marker for leukemic blast cells and its role in leukemogenesis." Methods Mol Med **68**: 223-37.

Svensson, E., H. Eriksson, et al. (2005). "DNA-binding dependent and independent functions of WT1 protein during human hematopoiesis." Exp Cell Res. **308**(1): 211-21.

Szczepanski, T., A. W. Langerak, et al. (2000). "T cell receptor gamma (TCRG) gene rearrangements in T cell acute lymphoblastic leukemia reflect 'end-stage' recombinations: implications for minimal residual disease monitoring." Leukemia **14**(7): 1208-14.

Szczepanski, T., A. Orfao, et al. (2001a). "Minimal residual disease in leukaemia patients." Lancet Oncol **2**(7): 409-17.

Szczepanski, T., M. J. Willemse, et al. (2001b). "Precursor-B-ALL with D(H)-J(H) gene rearrangements have an immature immunogenotype with a high frequency of oligoclonality and hyperdiploidy of chromosome 14." Leukemia **15**(9): 1415-23.

Szczepanski, T., T. Flohr, et al. (2002a). "Molecular monitoring of residual disease using antigen receptor genes in childhood acute lymphoblastic leukaemia." Best Pract Res Clin Haematol **15**(1): 37-57.

Szczepanski, T., M. J. Willemse, et al. (2002b). "Comparative analysis of Ig and TCR gene rearrangements at diagnosis and at relapse of childhood precursor-B-ALL provides improved strategies for selection of stable PCR targets for monitoring of minimal residual disease." Blood **99**(7): 2315-23.

Szczepanski, T., V. H. van der Velden, et al. (2003). "Comparative analysis of T-cell receptor gene rearrangements at diagnosis and relapse of T-cell acute lymphoblastic leukemia (T-ALL) shows high stability of clonal markers for monitoring of minimal residual disease and reveals the occurrence of second T-ALL." Leukemia. **17**(11): 2149-56.

Tafuri, A., C. Gregorj, et al. (2002). "MDR1 protein expression is an independent predictor of complete remission in newly diagnosed adult acute lymphoblastic leukemia." Blood **100**(3): 974-81.

Tamaki, H., M. Mishima, et al. (2003). "Monitoring minimal residual disease in leukemia using real-time quantitative polymerase chain reaction for Wilms tumor gene (WT1)." Int J Hematol **78**(4): 349-56.

Testa, U., G. F. Torelli, et al. (2002). "Human acute stem cell leukemia with multilineage differentiation potential via cascade activation of growth factor receptors." Blood **99**(12): 4634-7.

Thiede, C., C. Steudel, et al. (2002). "Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis." Blood **99**(12): 4326-35.

Thompson, A., M. F. Quinn, et al. (2003). "Global down-regulation of HOX gene expression in PML-RARalpha + acute promyelocytic leukemia identified by small-array real-time PCR." Blood **101**(4): 1558-65.

Thorsteinsdottir, U., E. Kroon, et al. (2001). "Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia." Mol Cell Biol **21**(1): 224-34.

Tiensiwakul, P., T. Lertlum, et al. (1999). "Immunophenotyping of acute lymphoblastic leukemia in pediatric patients by three-color flow cytometric analysis." Asian Pac J Allergy Immunol **17**(1): 17-21.

Tobal, K., J. Newton, et al. (2000). "Molecular quantitation of minimal residual disease in acute myeloid leukemia with t(8;21) can identify patients in durable remission and predict clinical relapse." Blood **95**(3): 815-9.

Trka, J., M. Kalinova, et al. (2002). "Real-time quantitative PCR detection of WT1 gene expression in children with AML: prognostic significance, correlation with disease status and residual disease detection by flow cytometry." Leukemia **16**(7): 1381-9.

Tschan, M. P., S. Vonlanthen, et al. (2001). "Different p16INK4a and p14ARF expression patterns in acute myeloid leukaemia and normal blood leukocytes." Leuk Lymphoma **42**(5): 1077-87.

Tse, K. F., J. Allebach, et al. (2002). "Inhibition of the transforming activity of FLT3 internal tandem duplication mutants from AML patients by a tyrosine kinase inhibitor." Leukemia **16**(10): 2027-36.

Tsuji, T., M. Yasukawa, et al. (2005). "Generation of tumor-specific, HLA class I-restricted human Th1 and Tc1 cells by cell engineering with tumor peptide-specific T-cell receptor genes." Blood. **106**(2): 470-6. Epub 2005 Mar 24.

Tumkaya, T., M. van der Burg, et al. (2001). "Immunoglobulin lambda isotype gene rearrangements in B cell malignancies." Leukemia **15**(1): 121-7.

Tutor, O., M. A. Diaz, et al. (2002). "Loss of heterozygosity of p16 correlates with minimal residual disease at the end of the induction therapy in non-high risk childhood B-cell precursor acute lymphoblastic leukemia." Leuk Res **26**(9): 817-20.

Uzunel, M., J. Mattsson, et al. (2001). "The significance of graft-versus-host disease and pretransplantation minimal residual disease status to outcome after allogeneic stem cell transplantation in patients with acute lymphoblastic leukemia." Blood **98**(6): 1982-4.

Uzunel, M., M. Jaksch, et al. (2003). "Minimal residual disease detection after allogeneic stem cell transplantation is correlated to relapse in patients with acute lymphoblastic leukaemia." Br J Haematol **122**(5): 788-94.

Uzunel, M., O. Ringden, et al. (2004). "Poor correlation of kinetics between BCR-ABL and WT1 transcript levels after allogeneic stem cell transplantation." Bone Marrow Transplant **33**(1): 47-52.

Vajjhala, P. R., E. Macmillan, et al. (2003). "The Wilms' tumour suppressor protein, WT1, undergoes CRM1-independent nucleocytoplasmic shuttling." FEBS Lett **554**(1-2): 143-8.

van Denderen, J., P. ten Hacken, et al. (1994). "Recognition of the ALL-specific BCR-ABL junction in P190bcr-abl by monoclonal antibody ER-FP1." Leukemia **8**(9): 1503-9.

van der Reijden, B. A., A. Simons, et al. (2002). "Minimal residual disease quantification in patients with acute myeloid leukaemia and inv(16)/CBFB-MYH11 gene fusion." Br J Haematol **118**(2): 411-8.

van der Velden, V. H., S. A. Joosten, et al. (2001). "Real-time quantitative PCR for detection of minimal residual disease before allogeneic stem cell transplantation predicts outcome in children with acute lymphoblastic leukemia." Leukemia **15**(9): 1485-7.

van der Velden, V. H., D. C. Jacobs, et al. (2002a). "Minimal residual disease levels in bone marrow and peripheral blood are comparable in children with T cell acute lymphoblastic leukemia (ALL), but not in precursor-B-ALL." Leukemia **16**(8): 1432-6.

van der Velden, V. H., J. M. Wijkhuijs, et al. (2002b). "T cell receptor gamma gene rearrangements as targets for detection of minimal residual disease in acute lymphoblastic leukemia by real-time quantitative PCR analysis." Leukemia **16**(7): 1372-80.

van der Velden, V. H., M. J. Willemse, et al. (2002c). "Immunoglobulin kappa deleting element rearrangements in precursor-B acute lymphoblastic leukemia are stable targets for detection of minimal residual disease by real-time quantitative PCR." Leukemia **16**(5): 928-36.

van der Velden, V. H., A. Hochhaus, et al. (2003). "Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects." Leukemia **17**(6): 1013-34.

van der Velden, V. H., N. Boeckx, et al. (2004a). "Differential stability of control gene and fusion gene transcripts over time may hamper accurate quantification of minimal residual disease--a study within the Europe Against Cancer Program." Leukemia **18**(4): 884-6.

van der Velden, V. H., N. Boeckx, et al. (2004b). "Detection of minimal residual disease in acute leukemia." J Biol Regul Homeost Agents. **18**(2): 146-54.

van Dongen, J. J., T. Seriu, et al. (1998). "Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood." Lancet **352**(9142): 1731-8.

van Dongen, J. J., E. A. Macintyre, et al. (1999). "Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia." Leukemia **13**(12): 1901-28.

van Dongen, J. J., A. W. Langerak, et al. (2003). "Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936." Leukemia **17**(12): 2257-317.

van Lochem, E. G., Y. M. Wiegers, et al. (2000). "Regeneration pattern of precursor-B-cells in bone marrow of acute lymphoblastic leukemia patients depends on the type of preceding chemotherapy." Leukemia **14**(4): 688-95.

van Oostveen, J., J. Bijl, et al. (1999). "The role of homeobox genes in normal hematopoiesis and hematological malignancies." Leukemia **13**(11): 1675-90.

Van Waalwijk Van Doorn-Khosrovani, S. B., C. Erpelinck, et al. (2003). "Biallelic mutations in the CEBPA gene and low CEBPA expression levels as prognostic markers in intermediate-risk AML." Hematol J **4**(1): 31-40.

van Wering, E. R., B. E. van der Linden-Schrever, et al. (2000). "Regenerating normal B-cell precursors during and after treatment of acute lymphoblastic leukaemia: implications for monitoring of minimal residual disease." Br J Haematol **110**(1): 139-46.

Veltroni, M., L. De Zen, et al. (2003). "Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: implications for the detection of minimal residual disease in acute lymphocytic leukemia." Haematologica **88**(11): 1245-52.

Venditti, A., F. Buccisano, et al. (2000). "Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia." Blood **96**(12): 3948-52.

Venditti, A., L. Maurillo, et al. (2003). "Pretransplant minimal residual disease level predicts clinical outcome in patients with acute myeloid leukemia receiving high-dose chemotherapy and autologous stem cell transplantation." Leukemia **17**(11): 2178-82.

Verhagen, O. J., M. J. Willemse, et al. (2000). "Application of germline IGH probes in real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia." Leukemia **14**(8): 1426-35.

Vial, J. P. and F. Lacombe (2001). "Immunophenotyping of acute leukemia: utility of CD45 for blast cell identification." Methods Cell Biol **64**: 343-58.

Vidriales, M. B., J. F. San-Miguel, et al. (2003). "Minimal residual disease monitoring by flow cytometry." Best Pract Res Clin Haematol **16**(4): 599-612.

Wagner, K. D., N. Wagner, et al. (2003). "The complex life of WT1." J Cell Sci **116**(Pt 9): 1653-8.

Weisser, M., W. Kern, et al. (2005). "Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia." Leukemia. **19**(8): 1416-23.

Westman, B. J., J. P. Mackay, et al. (2002). "Ikaros: a key regulator of haematopoiesis." Int J Biochem Cell Biol **34**(10): 1304-7.

Willemse, M. J., T. Seriu, et al. (2002). "Detection of minimal residual disease identifies differences in treatment response between T-ALL and precursor B-ALL." Blood **99**(12): 4386-93.

Wong, I. H., M. H. Ng, et al. (2000). "Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications." Blood **95**(6): 1942-9.

Wuchter, C., J. Harbott, et al. (2000). "Detection of acute leukemia cells with mixed lineage leukemia (MLL) gene rearrangements by flow cytometry using monoclonal antibody 7.1." Leukemia **14**(7): 1232-8.

Xu, F., T. Taki, et al. (2000). "Tandem duplication of the FLT3 gene is infrequent in infant acute leukemia. Japan Infant Leukemia Study Group." Leukemia **14**(5): 945-7.

Yamaguchi, H., K. Inokuchi, et al. (2002). "The study for loss of bcl-xs expression as a prognostic factor in acute myeloid leukemia." Leuk Res **26**(12): 1119-23.

Yamamoto, Y., H. Kiyoi, et al. (2001). "Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies." Blood **97**(8): 2434-9.

Ye, Y., B. Raychaudhuri, et al. (1996). "Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation." Embo J **15**(20): 5606-15.

Yee, K. W., A. M. O'Farrell, et al. (2002). "SU5416 and SU5614 inhibit kinase activity of wild-type and mutant FLT3 receptor tyrosine kinase." Blood **100**(8): 2941-9.

Zamai, L., B. Canonico, et al. (2002). "Intracellular detection of Bcl-2 and p53 proteins by flow cytometry: comparison of monoclonal antibodies and sample preparation protocols." J Biol Regul Homeost Agents **16**(4): 289-302.

Zhang, X., G. Xing, et al. (1997). "Transactivation of an intronic hematopoietic-specific enhancer of the human Wilms' tumor 1 gene by GATA-1 and c-Myb." J Biol Chem **272**(46): 29272-80.

Zhang, Q. Y., K. Garner, et al. (2002). "Rapid detection of leukemia-associated translocation fusion genes using a novel combined RT-PCR and flow cytometric method." Leukemia **16**(1): 144-9.

Zuna, J., K. Muzikova, et al. (2002). "Significance of real-time quantitative PCR detection of p16 gene deletions in childhood acute lymphoblastic leukemia." Haematologica **87**(6): 668-9.

Zwaan, C. M., S. Meshinchi, et al. (2003). "FLT3 internal tandem duplication in 234 children with acute myeloid leukemia (AML): prognostic significance and relation to cellular drug resistance." Blood **19**: 19.